STUDIES ON THE REGULATION BY CALCIUM AND CALCIUM-MOBILIZING HORMONES OF BILE FLOW IN RAT LIVER

by

ARI ENSIO KARJALAINEN

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Statement

The design, execution (apart from assistance of a technical nature in aspects of some experiments) and interpretation of all experiments in this thesis was generally carried out by myself but involved discussions with my supervisor. Some of the experiments involving cholestasis were begun with Dr. Yuhki Hamada.

Ari E. Karjalainen

Acknowledgments

I would like to thank Dr. Maurice J. Weidemann for giving me the opportunity to undertake this work in the division, and successive heads of the division, Professor Christopher Bryant and Dr. David Day for their helpfulness during this time.

I would like to extend my sincerest gratitude to my supervisor Dr. Fyfe Bygrave, for the opportunity to be involved in this project, and for the advice, encouragement, friendship and support which were extended over the period of the studentship. These, along with helpful discussions resulted in the creation of an atmosphere that is conducive to research.

I am also thankful to Dr. Yuhki Hamada (Visiting Fellow), who initially raised some of the propositions which are central to this thesis, and whose enthusiasm for the work was infectious. Helpful discussions and comments from him, as well as with Dr. Elizabeth Milbourne (Research Officer), my co-supervisors, Dr. Peter Janssens and Dr. Paul Waring, and Tanya Applegate (honours student) are acknowledged. My thanks are also extended to Jamie Baker, Sue Bedford, Richard Conan-Davies, Jim Krayshek, Jacqueline Millard, Helen Roberts and Damon Shorter who, at one time or another, have given excellent technical help in aspects of some of the experiments. The advice and assistance of the senior technical officer Mr. Bill Nicholson is also gratefully acknowledged.

I am grateful to my wife Tarja for her patience, support and assistance during the studentship, which has made this work possible.

Publications arising out of the work for this thesis

1. Karjalainen, A. & Bygrave, F. L. (1994).

The synergistic action (cross-talk) of glucagon and vasopressin induces early bile flow and plasma-membrane calcium fluxes in the perfused rat liver.

Biochemical Journal, 301, 187-192.

2. Bygrave, F. L., Karjalainen, A. & Hamada, Y. (1994).

Cross-talk between calcium- and cyclic AMP-mediated signalling systems and the short-term modulation of bile flow in normal and cholestatic rat liver.

Cellular Signalling, 6, 1-9.

3. Hamada, Y., Karjalainen, A. & Bygrave, F. L. (1995).

Hormone-induced bile flow and hepatobiliary calcium fluxes are attenuated in the perfused liver of rats made cholestatic with ethynylestradiol *in vivo* and with phalloidin *in vitro*.

Hepatology, 21, 1455-1464.

4. Karjalainen, A. & Bygrave, F. L. (1995).

Nickel: an agent for investigating the relation between hormone-induced Ca^{2+} influx and bile flow in the perfused rat liver.

Cell Calcium, 18, 214-222.

Outline of presentation

The major sections of this thesis consist of the Introductory Review, the Experimental Section and the General Discussion. The Introductory Review presents an overview of the current state of knowledge relating to the role of calcium and calcium-mobilizing hormones in liver, and consists of two parts. The focus of Part 1 is on the mechanisms involved in the hormonal regulation of Ca^{2+} homeostasis. The literature on the mechanisms by which glucagon and the Ca^{2+} mobilizing hormones influence bile flow events are covered in Part 2. The review is followed by a statement of the aims of this present study.

The Experimental Section consists of Chapters where the results of these experiments are presented, along with the major conclusions for each section of work. The order of presentation is not necessarily the order in which the experiments were carried out. This section is preceded by a General Methods section, which contains a description of those methods which are common to all chapters. Any methods which are unique to a chapter are present in the context of that chapter.

The General Discussion draws together the data and interpretations obtained during this study, and the relationship of these to the current state of knowledge of the regulation of Ca^{2+} fluxes and bile flow is defined. Directions for further research are identified which seem to be of immediate importance for furthering of understanding in the field.

Each figure legend and table legend faces the figure or table and follows the page on which reference to it is first made.

Abbreviations

Chemicals

ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BHQ	2,5-di-*tert*-butyl-hydroquinone
Ca^{2+}	'free' calcium ion
Ni^{2+}	'free' nickel ion
cyclic AMP	cyclic adenosine 3',5'-monophosphate
G-protein	Guanosine triphosphate (GTP)-binding protein
IP_3	Inositol 1,4,5-trisphosphate
IP_4	Inositol 1,3,4,5-tetrakisphosphate
G-UDCA	Glyco-Ursodeoxycholic Acid
T-CDCA	Tauro-Chenodeoxycholic Acid
T-UDCA	Tauro-Ursodeoxycholic Acid
UDCA	Ursodeoxycholic Acid

Units

min	minutes
s	seconds
µg, mg or g	micrograms, milligrams or grams respectively
µl or ml	micro- or millilitres respectively
nM, µM or mM	nano-, micro- or millimolar respectively

Any other units are defined in the text, at the point where they first occur.

Table of Contents

Section A

ABSTRACT

ABSTRACT

This work describes investigations into the mechanism by which inflow of Ca^{2+} into the perfused rat liver is regulated by cross-talk between the phosphoinositide and cyclic AMP signalling pathways, and the modulation of bile flow induced by this cross-talk.

Examination of the short-term hormone-induced Ca^{2+} fluxes and bile flow events revealed that the agonist-specificity in their potential for stimulation of Ca^{2+} influx was reflected in the extent to which bile flow was stimulated. Furthermore, potentiation of this influx in the presence of glucagon was accompanied by a synergistic stimulation of bile flow. This correlation between Ca^{2+} influx and bile flow was also reflected in the timing of these responses.

Glucagon plus vasopressin-mediated synergistic influx of Ca^{2+} was inhibited by added Ni^{2+} in a concentration-dependent manner. This identified an optimum state of Ca^{2+} flux for bile secretion, and provided indications for a balance between stimulatory and inhibitory actions of Ca^{2+} on this event, and suggests that the localization of the increases in intracellular Ca^{2+} determine the extent to which the events are stimulated.

The greater sensitivity for efflux of Ca^{2+} from the liver was indicated by the temporal relationship between net efflux and influx, as well as the relative dependence of these events on the concentration of the agonists. Sensitization for influx by glucagon was reflected in the stimulation of bile flow. Indications are that the synergistic stimulation of the flow of bile might, in part, involve a further interaction between cyclic AMP generated and the increased Ca^{2+} flux induced by this messenger in the presence of the Ca^{2+}-mobilizing agonists.

The second part of this work concentrated on the relationship between hepatobiliary Ca^{2+} fluxes and bile flow in cholestasis induced by the administration of ethynylestradiol in vivo or phalloidin in vitro. A prominent feature of cholestasis was the attenuation of the agonist-mediated increase in bile flow. Attenuation of Ca^{2+} fluxes also accompanied cholestasis, but was dependent on the method by which cholestasis was induced. These differences identified further tools for studying the mechanism of

bile flow, Ca^{2+} influx and the role of hormones in these. Furthermore, this work sets the scene for the elucidation of treatments which overcome the lesions in agonist-stimulated bile flow, indicating the therapeutic potential of these studies for cholestasis.

The effects were investigated of choleretic bile acids on the flow of bile and of hepato-biliary calcium induced by the short-term administration of vasopressin and glucagon in experimentally-induced cholestasis. While bile flow was increased in the presence of these agents, the pattern of the response to stimulation by hormones was less affected. Thus acute treatment with bile acids in these experiments relieves the primary symptom (bile flow) to a greater extent than the underlying lesion in these models.

Section B

INTRODUCTORY REVIEW

INTRODUCTORY REVIEW

PART 1

PART 2

(A) Introduction to the thesis

This introduction reviews recent findings concerning the mechanism by which hormones mobilize Ca^{2+} in liver cells and thereby modulate bile flow. Because the author's work comprises results obtained using the perfused rat liver, a brief description of this system is given at the outset, along with an overview of relevant liver anatomy and physiology. The perfused liver system enables measurement of both bile flow and perfusate Ca^{2+} changes induced by a range of stimuli including glucagon as well as the Ca^{2+}-mobilizing agonists. These changes, which can be monitored continuously and simultaneously, reflect events occurring at opposite poles of the liver cell. This system thus permits a temporal and quantitative correlation between these events to be made. The relationship between bile flow and Ca^{2+} mobilization suggests that this process may be subject to physiological modulation by Ca^{2+}. Conversely, induction of intrahepatic cholestasis can in some situations affect Ca^{2+} fluxes induced by these agents. Thus the first part of the review deals with the mechanisms by which the interaction of a Ca^{2+}-mobilizing agonist such as vasopressin with its receptor, is able to induce Ca^{2+} fluxes in liver cells. The interaction of these agonists with their receptors stimulates a reaction cascade that results in the formation of products of phosphoinositide hydrolysis. These compounds, known as 'second messengers', in turn stimulate the release of Ca^{2+} from intracellular storage sites and under certain conditions, induce influx of Ca^{2+} from the extracellular medium. These events are accompanied by the stimulation of protein kinase C activity through its interaction with diacylglycerol, another by-product of phosphoinositide hydrolysis. The mechanisms by which these events take place are described. Particular emphasis is given to the mechanism by which "cross-talk" between cyclic AMP-generating signalling cascades and pathways involved in phosphoinositide hydrolysis occurs to synergistically induce Ca^{2+} influx.

The second part of the review deals with the mechanisms involved in the regulation of bile flow. The effects of Ca^{2+} on components of the hepatocyte, be they structural or metabolic, that are implicated in bile flow receive special attention. An important determinant of the rate of bile flow is the balance between cholestatic and choleretic bile acids in bile. The role of Ca^{2+} in

aspects of uptake, intracellular transport, modification and synthesis, as well as release of bile acids into the canaliculus thus is dealt with. In addition to their role in directly modulating bile flow, many bile acids are able to stimulate both influx and efflux of Ca^{2+} and also modify actions of Ca^{2+}-mobilizing hormones; the mechanisms by which they are thought to do so form a part of this review. Differences between the effects of cholestatic and choleretic bile acids on Ca^{2+} mobilization are discussed as is the question of the relevance of this Ca^{2+} mobilization profile to their effect on bile flow. Bile flow can involve contractile, secretory, osmotic, metabolic and permeability elements, and the rate at which this occurs at any given moment is ultimately the sum of many individual factors. Thus an intricate interplay of components of intracellular architecture are very likely involved. Biochemical lesions can occur at numerous points, resulting in a decreased bile flow rate, a condition referred to as cholestasis. Various organelles and vesicular structures have been implicated in the pathology of cholestasis, as well as cytoskeletal elements, including molecular 'motors', membrane transporters and enzymes. Because components of the phosphoinositide-Ca^{2+} and cyclic AMP-signalling pathways regulate the activity of many of these processes, the number of putative sites of modulation of bile flow by these is considerable.

While extracellular Ca^{2+} is obligatory for bile flow to occur, the concentration of Ca^{2+} in the cytosol seems poised between stimulation and inhibition of bile flow. Thus recent observations of the wave-like nature of agonist-mediated changes in intracellular Ca^{2+} concentration in cells, including hepatocytes, may provide clues for an integrated hypothesis for mechanism(s) of bile flow. The nature, physical location and characteristics of regulation of the release of Ca^{2+} into the cytosol of the cell determine the Ca^{2+} "status" at a physical point at any given time. This also determines the state of activation of the many physiological functions which are subject to regulation by Ca^{2+}. Thus spatio-temporal aspects of Ca^{2+} fluxes also might be an important additional factor in the modulation, by cross-talk, between the signalling systems involving phosphoinositide hydrolysis and cyclic AMP-induced production and release of bile. The mechanism by which cross-talk between these signalling pathways results in synergistic effects in both Ca^{2+}-mobilization and bile flow, and how these are altered in cholestasis,

forms the thrust of this review. A proposal will be put, whereby the Ca^{2+} fluxes are treated as wave-like phenomena, and where the path traversed by a quantum of the ion determines the type of factors contributory to bile flow, as well as the extent to which these factors are stimulated or inhibited.

(B) The perfused liver system

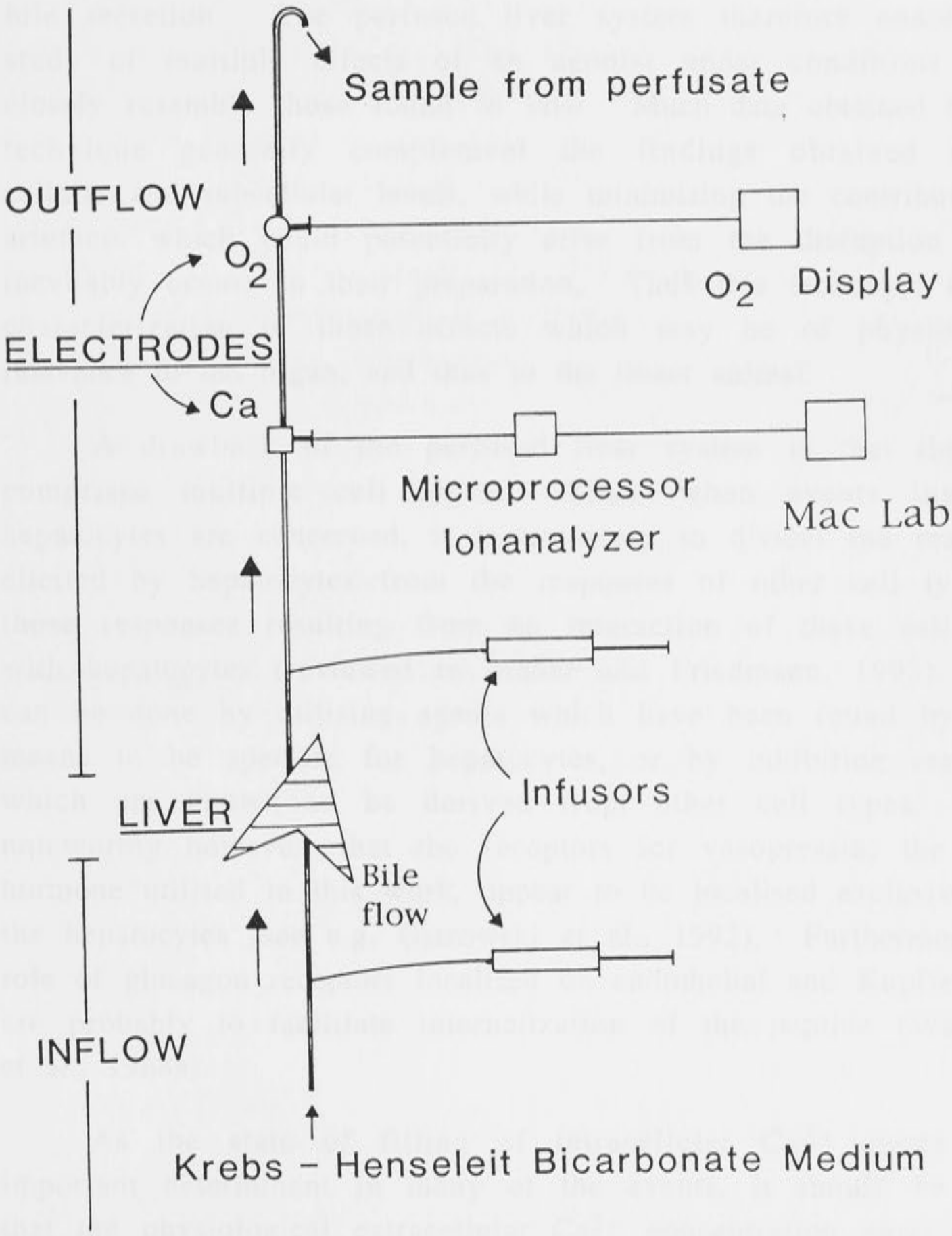
Studies using the perfused rat liver coupled to a continuous-flow Ca^{2+} electrode system have contributed significantly to our understanding of the mechanism of action of Ca^{2+}-mobilizing agonists, as well as to the physiological responses elicited by these agents. In characterizing the effects of Ca^{2+} on a physiological event such as bile flow, which involves the whole organ, this system is particularly useful. Since much of the work reviewed herein consists of results largely obtained with the perfused liver, a brief description of this experimental system follows (for further detail see Reinhart et al., 1982a; Altin and Bygrave, 1985; Altin, 1988, and for a review see Bygrave, 1995).

The perfused liver essentially involves an experimental system in which the portal blood supply to the liver is replaced with a physiological medium such as Krebs-Henseleit bicarbonate buffer (Krebs and Henseleit, 1932) (Fig.1). The medium is maintained at 37°C and oxygenated with a mixture of O_2/CO_2 (19:1); under these conditions the pH is maintained at 7.4. The liver remains metabolically viable, even with the basic perfusion medium described above, for over two hours. The effluent medium collected from the vena cava is passed through a chamber containing electrodes for measuring the concentrations of components of the medium such as Ca^{2+} and O_2. Agents can be infused into the portal vein by pump-driven syringes for a determined length of time during an experiment. Changes in perfusate Ca^{2+}, which reflect Ca^{2+} influx or efflux across the plasma membrane of liver cells, can be measured continuously at high resolution even in the presence of a physiological Ca^{2+} concentration. This enables the temporal relationship between these events to be determined precisely. Because the infusion of agents can be rapidly terminated, the resultant responses can be analysed also. After passing through the electrodes the effluent perfusate can be collected for further analysis. For example, an increase in the release of glucose into the perfusate can act as a further indicator of an increase in intracellular Ca^{2+} that occurs in hepatocytes, as well as serving as one reliable indicator of the viability of the liver.

Fig.1. The perfused rat liver system

Components of the perfused rat liver system are shown. As indicated in the text, livers are routinely perfused with oxygenated Krebs-Henseleit bicarbonate buffer containing 1.3mM Ca^{2+} at 37°C. Changes in perfusate calcium and oxygen concentration in response to infusion of agents are continuously monitored by utilizing the appropriate electrodes. Perfusate may be collected at the outflow point for further analysis. Cannulation of the bile duct enables ready monitoring of bile flow rate as well as composition. For further details see text.

PERFUSED RAT LIVER SYSTEM



Further physiological data can be gained by inserting a cannula into the bile duct, and by collecting the bile into pre-tared tubes at specified intervals of time. By this technique, meaningful data have been obtained in this work even when samples of bile have been collected at intervals as short as 5 seconds. This enables correlations between Ca^{2+} and bile flow events to be made at considerable resolution. Subsequent further analyses of the bile sample for its components, such as total calcium or bile acids, can also yield important data relevant to the mechanisms involved in bile secretion. The perfused liver system therefore enables the study of multiple effects of an agonist under conditions which closely resemble those found *in vivo*. Much data obtained by this technique generally complement the findings obtained at the cellular and subcellular levels, while minimising the contribution of artefacts which could potentially arise from the disruption which inevitably occurs in their preparation. Thus this technique enables characterization of those effects which may be of physiological relevance to the organ, and thus to the intact animal.

A drawback of the perfused liver system is that the liver comprises multiple cell types. Thus, when events involving hepatocytes are concerned, it is necessary to dissect the responses elicited by hepatocytes from the responses of other cell types or those responses resulting from an interaction of these cell types with hepatocytes (reviewed in Maher and Friedmann, 1993). This can be done by utilising agents which have been found by other means to be specific for hepatocytes, or by inhibiting responses which are known to be derived from other cell types. It is noteworthy however, that the receptors for vasopressin, the major hormone utilised in this work, appear to be localised exclusively on the hepatocytes (see e.g. Ostrowski et al., 1992). Furthermore, the role of glucagon receptors localised on endothelial and Kupffer cells are probably to facilitate internalization of the peptide (Watanabe et al., 1988a).

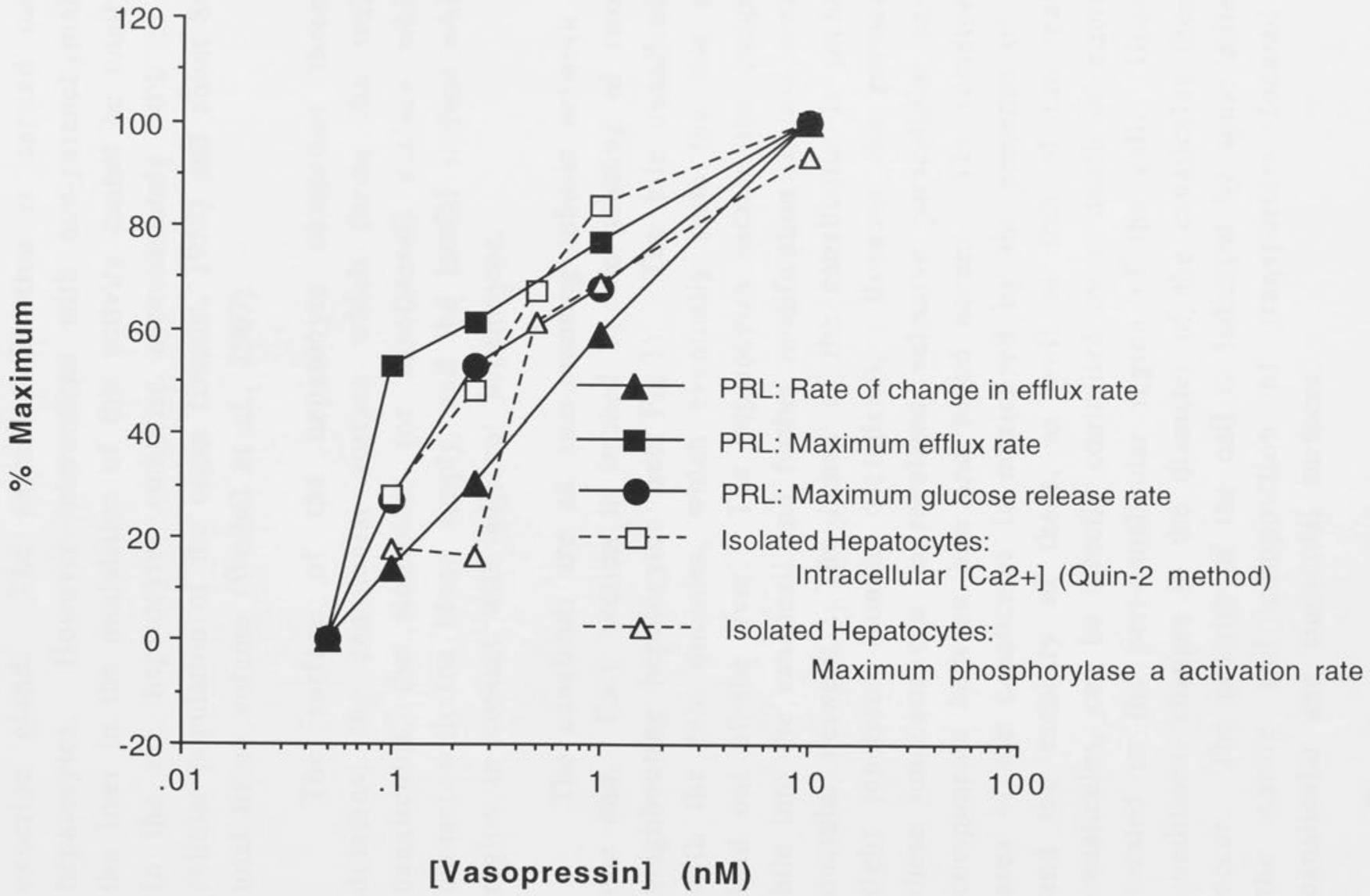
As the state of filling of intracellular Ca^{2+} stores is an important determinant in many of the events, it should be noted that the physiological extracellular Ca^{2+} concentration employed in these studies has ensured that the Ca^{2+} stores in the liver should be filled at the moment when the agonist(s) are applied. The maximum rate of Ca^{2+} efflux as well as the acceleration in that rate

are related to intracellular Ca^{2+} mobilization. The former reflects the increased activity of the plasma membrane Ca^{2+},Mg^{2+}-ATPase, which is stimulated to extrude the ion, and which in turn is related to the intracellular Ca^{2+} concentration. The acceleration of this rate would reflect the rate at which these Ca^{2+}-ATPases are activated within the liver (see e.g. Epping and Bygrave, 1984).

The minimum concentration of vasopressin at which the release of Ca^{2+} from the perfused rat liver is observed in this work coincide with data where an increase in the intracellular Ca^{2+} concentration (Thomas et al., 1984; Charest et al., 1985; Lynch et al., 1985; Combettes et al., 1986) or oscillations of Ca^{2+} concentration are induced (Rooney et al., 1989; Sanchez-Bueno et al., 1993) in hepatocytes. Under these conditions the rate of glucose release reflects the Ca^{2+}-mediated activation of glycogen phosphorylase, and therefore reflects changes in intracellular Ca^{2+} concentration. The rate of glucose release is proportional to the concentration of vasopressin, and the observations described herein are consistent with the changes in intracellular Ca^{2+} measured with other methods in hepatocytes (e.g. Thomas et al., 1984) (Fig.2). The fact that there are substantial variations in the data obtained by these techniques at the low concentrations of the hormones (see Fig.2) is also reflected in the considerable differences in these parameters with isolated hepatocytes. However, the similarity of data between these systems has recently been illustrated by the concentration-dependence of changes in the rate of oscillations in intracellular Ca^{2+} in the perfused rat liver (Robb-Gaspers and Thomas, 1995; Thomas et al., 1995).

Fig.2. The effect of vasopressin on Ca^{2+} fluxes.

A comparison of the vasopressin concentration dependence of events which reflect changes in intracellular Ca^{2+} concentration with the perfused liver and isolated hepatocytes. Initial changes in the intracellular Ca^{2+} concentration is reflected in the perfused liver by the maximal efflux rate as well as the maximum acceleration of that rate. The rate of glucose release reflects the extent of stimulation of glycogen phosphorylase in the perfused rat liver, and the initial rate of activation of this enzyme can be measured in the hepatocytes. Direct monitoring of the intracellular Ca^{2+} concentration is readily performed in the isolated hepatocytes by utilizing Ca^{2+}-sensitive dyes such as quin-2. The hepatocyte data was compiled from data presented in Thomas et al. (1984), Charest et al. (1985), Lynch et al. (1985) and Combettes et al. (1986). The perfused liver data was derived from experiments for Fig.1, Chapter 3, Section E. See text for further details.


120
100
80
60
40
20
0
-20
.01
.1
1
10
100
% Maximum
[Vasopressin] (nM)
PRL: Rate of change in efflux rate
PRL: Maximum efflux rate
PRL: Maximum glucose release rate
Isolated Hepatocytes:
Intracellular [Ca2+] (Quin-2 method)
Isolated Hepatocytes:
Maximum phosphorylase a activation rate

(C) Overview of relevant liver anatomy

Because an important aspect of the work in this thesis concerns the secretory function of the liver, this section of the review will concentrate largely on the role of this organ as an exocrine gland. The secretory function is carried out by the hepatocytes. However interactions with non-parenchymal cells of the liver in the modulation of this activity cannot be totally ignored. In the rat, hepatocytes comprise approximately 60% of the total cellular population of the organ (Daoust, 1958) and about 80% of the total tissue volume (Weibel et al., 1967).

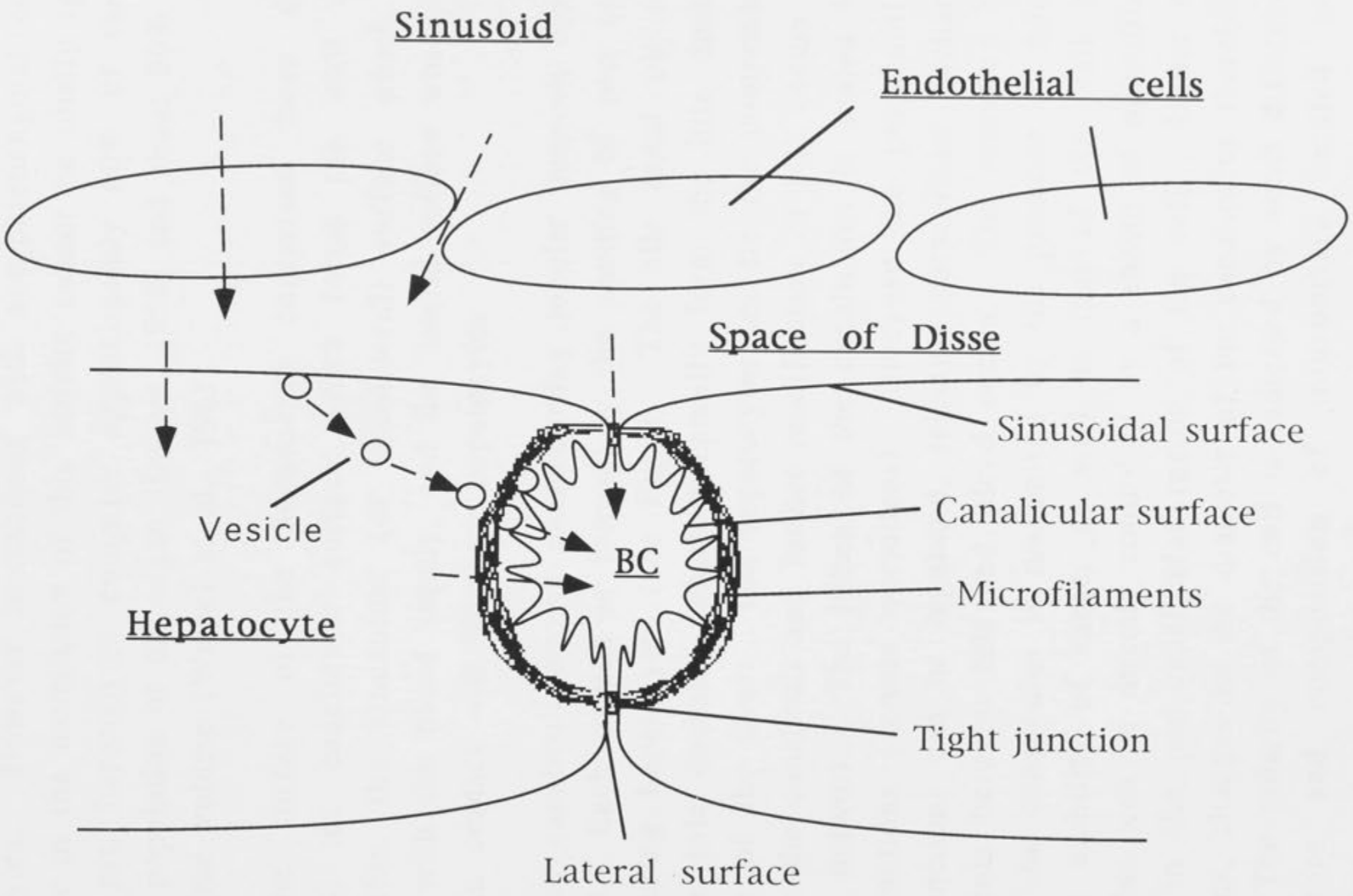
The surface of the hepatocyte comprises three distinct domains; the canalicular surface which forms the wall of the canaliculus, the sinusoidal (or basolateral) surface which is in contact with the blood supply, and the lateral surface which is the region in contact with adjacent hepatocytes.

The canaliculi are an anastomosing tubular network closed at one end. Each section is formed by the abutting of two or three neighbouring hepatocytes (see Fig.3). The bile canaliculi connect with the bile ductules, which eventually form the bile ducts that lead out of the liver. The hepatocytes secrete the components of bile into the canaliculi and further modification of bile occurs in the ductular network. The lumen of the canaliculus is sealed by the tight junctions (zonula occludens). Because the permeability of these junctions can be regulated, selective paracellular exchange of components between bile and blood occurs. The secretory surface area of the canaliculus is maximised by the presence of microvilli and the mobility of these, as well as that of the wall of the canaliculus, can be directly controlled by a sheath of microfilaments located in the peri-canalicular region of the cell. Under certain conditions, changes in the diameter of the canaliculus therefore can occur. The polarity of the cell is indicated by some differences in the nature and composition of transporters located on the canalicular and sinusoidal surfaces.

The hepatocytes are generally arranged in plates of single-cell thickness such that the basolateral surfaces of the cell are oriented towards the sinusoidal space, as shown in the figure. The porosity of the epithelium which lines the hepatocytes results from the loose

Fig.3. Anatomy of the hepatocytes.

The main features of mechanisms involved in the transfer of material from the sinusoids (blood) to the bile canaliculus (BC) (arrows) and in the secretion of bile. Non-cellular components reach the hepatocyte by passing through the endothelial layer into the Space of Disse. Transport of components to the bile can then occur either transcellularly, by diffusion through the cell, bound to transport proteins or by vesicular movements. Exchange of components between blood and bile can also occur paracellularly, through the tight junctions. Contraction of the microfilaments which form the pericanalicular sheath results in the contraction of the canalicular lumen. See the text for further details.


Sinusoid
Endothelial cells
Space of Disse
Sinusoidal surface
Canalicular surface
Microfilaments
Tight junction
Lateral surface
Vesicle
BC
Hepatocyte

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connections between the endothelial cells as well as fenestrations in the endothelial cells. This enables plasma, but not the cellular components of blood, to enter the Space of Disse. This relatively direct interaction of the hepatocytes with the blood supply, as well as the presence of numerous microvilli on the sinusoidal surface, ensures that the efficiency of metabolic exchange between the blood and hepatocytes is maximised. Transcellular passage of components of bile from the sinusoid to the canaliculus can involve microtubule-dependent vesicle transfer as well as passive diffusion.

The lateral surface comprises the region which abuts the adjoining hepatocytes. Therefore the prominent features of this region are structures associated with inter-cell adhesion, namely the desmosomes, intermediate junctions (zonula adherens) and gap junctions. The latter also serve to facilitate inter-cell communication, contributing to the co-ordination of responses throughout the organ. It has been shown that the hepatocytes are themselves a heterogeneous population of cells, with periportal and perivenous regions of the liver showing differences in enzyme, transporter and organellar distribution (for a review see e.g. Katz and Jungermann, 1993). The consequences of these, as well as the effect of a periportal to perivenous gradient in components of blood relevant for the flow of bile are not to be underestimated, but are beyond the scope of the present review.

INTRODUCTORY REVIEW

PART 1

The regulation of Ca^{2+} fluxes in rat liver

(D) The regulation of Ca^{2+} fluxes in rat liver

(I) Introductory overview

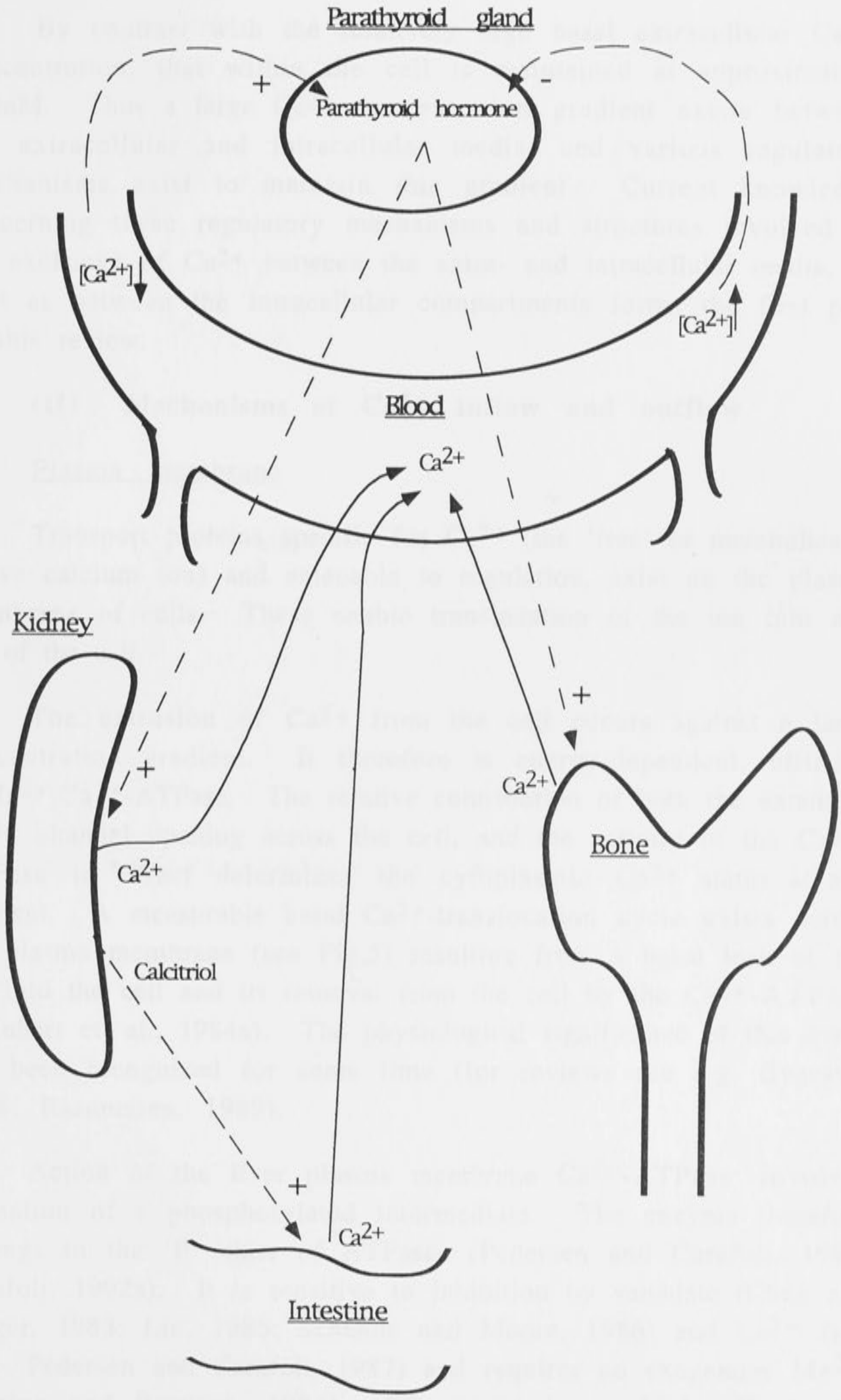
Ca^{2+} is essential for 'life', influencing a wide array of cellular functions. Apart from its universal physiological role in maintaining membrane integrity, a necessity for all life forms, it has an ubiquitous role in cellular signalling in eukaryotic cells. The mechanism by which its concentration is regulated in the extracellular environment, and even more importantly, within the intracellular medium, is thus of considerable significance. As indicated by Barritt (1992), the extracellular pool of Ca^{2+} can be viewed both as a limitless source of Ca^{2+} for cells, as well as an unquenchable sink for the ion. In view of the importance of the exchange of Ca^{2+} between the intracellular and extracellular compartments, an overview of the mechanisms involved in regulating the extracellular Ca^{2+} concentration will be presented below.

The extracellular medium of hepatocytes consists of the (blood) plasma at the sinusoidal and lateral domains, and bile fluid at the canalicular domain. Thus approximately 85% of the surface area of the cell is in contact with plasma, and the remainder with canalicular bile. It could be deduced from this that the plasma would provide the major extracellular contribution to intracellular Ca^{2+} homeostasis. However, as will be discussed, Ca^{2+} transport at the canalicular domain may also play a role.

The plasma Ca^{2+} concentration is tightly controlled at approximately 1.3 mM. The most important component of plasma Ca^{2+} regulation is the parathyroid hormone (see Fig.4) which induces a hypercalcaemic effect; its target organs are the bone and kidneys. Secretion of parathyroid hormone is itself regulated by the concentration of Ca^{2+} in the blood (reviewed in Nemeth, 1995). Parathyroid hormone stimulates reabsorption of Ca^{2+} from bone. The effect of the hormone on the kidney is twofold; (1) it induces distal tubular Ca^{2+} reabsorption, and (2) it stimulates the synthesis of 1,25-dihydroxy vitamin D (calcitriol). Calcitriol then stimulates an increase in intestinal Ca^{2+} absorption, tubular Ca^{2+} reabsorption in the kidneys and Ca^{2+} reabsorption from bone (reviewed in e.g.

Fig.4. Regulation of the extracellular Ca^{2+} concentration

The main effector is the parathyroid hormone, which is produced by the parathyroid gland in response to a decreased concentration of Ca^{2+} in the blood. The parathyroid hormone stimulates the reabsorption of Ca^{2+} from the bone and kidney, where it also stimulates the formation of calcitriol, which promotes the reabsorption of Ca^{2+} from the intestine. For further detail see text.


Parathyroid gland
+
-
Parathyroid hormone
[Ca2+]
[Ca2+]
Blood
Ca2+
Kidney
+
Ca2+
+
Ca2+
Bone
Calcitriol
+
Ca2+
Intestine

Aurbach, 1988; Schaafsma, 1988). In some species these functions are antagonized by the thyroid hormone calcitonin.

By contrast with the relatively high basal extracellular Ca^{2+} concentration, that within the cell is maintained at approximately 100nM. Thus a large Ca^{2+} concentration gradient exists between the extracellular and intracellular media, and various regulatory mechanisms exist to maintain this gradient. Current knowledge concerning these regulatory mechanisms and structures involved in the exchange of Ca^{2+} between the extra- and intracellular media, as well as between the intracellular compartments forms the first part of this review.

(II) Mechanisms of Ca^{2+} inflow and outflow

Plasma membrane

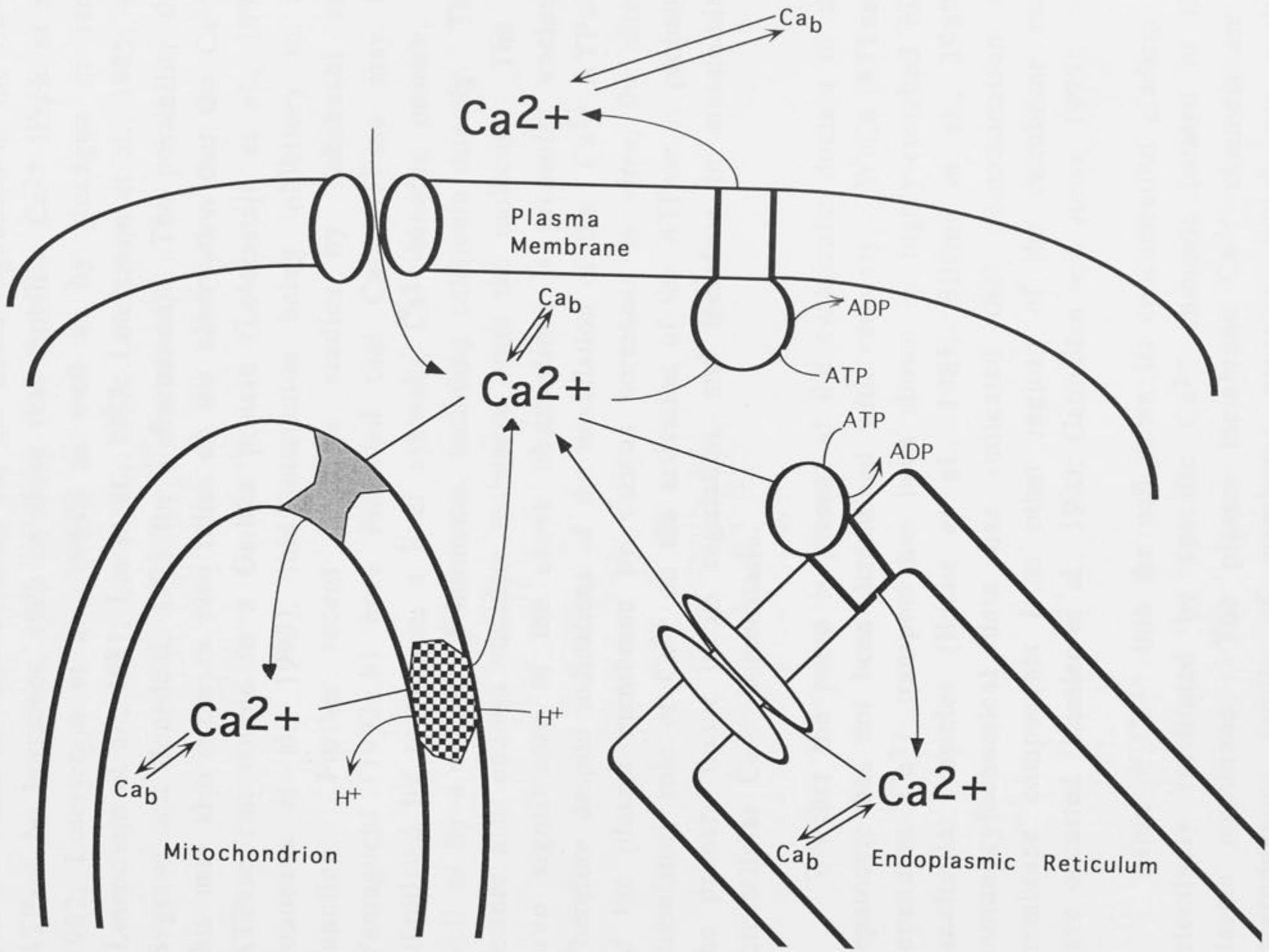
Transport proteins specific for Ca^{2+} (the 'free' or metabolically active calcium ion) and amenable to regulation, exist on the plasma membrane of cells. These enable translocation of the ion into and out of the cell.

The extrusion of Ca^{2+} from the cell occurs against a large concentration gradient. It therefore is energy-dependent, utilising a Mg^{2+},Ca^{2+}-ATPase. The relative contribution of both the extent of Ca^{2+} channel opening across the cell, and the activity of the Ca^{2+}-ATPase in effect determines the cytoplasmic Ca^{2+} status at any moment. A measurable basal Ca^{2+}-translocation cycle exists across the plasma membrane (see Fig.5) resulting from a basal leak of the ion into the cell and its removal from the cell by the Ca^{2+}-ATPase (Reinhart et. al., 1984a). The physiological significance of this event has been recognized for some time (for reviews see e.g. Bygrave, 1978; Rasmussen, 1989).

Action of the liver plasma membrane Ca^{2+}-ATPase involves formation of a phosphorylated intermediate. The enzyme therefore belongs to the 'P' class of ATPases (Pedersen and Carafoli, 1987; Carafoli, 1992a). It is sensitive to inhibition by vanadate (Chan and Junger, 1983; Lin, 1985; Schanne and Moore, 1986) and La^{3+} (see e.g. Pedersen and Carafoli, 1987) and requires no exogenous Mg^{2+} (Epping and Bygrave, 1984). The pump has a high affinity for calmodulin (K_m approximately 1nM), the binding of which increases

Fig.5. The cycling of Ca^{2+} across the membranes separating the domains of Ca^{2+}.

Influx of Ca^{2+} at the plasma membrane involves the regulated activation of the opening of specific channels for the ion. The activity of the ligand-gated channel on the endoplasmic reticular membrane is regulated by the binding of IP_3. Ca^{2+} is extruded from the cytosol into the extracellular medium, as well as into the endoplasmic reticulum by a Ca^{2+}-ATPase. Mitochondria sequester Ca^{2+} by the activity of a uniporter, and in the liver it is largely released as a result of the action of a Ca^{2+}-H^+-exchanger.


Ca b
Ca2+
Plasma
Membrane
Ca b
ADP
ATP
Ca2+
ATP
ADP
Ca2+
H+
Ca b
H+
Mitochondrion
Ca2+
Ca b
Endoplasmic Reticulum

its affinity for Ca^{2+} from 10-20μM to less than 0.5μM as well as increasing its activity approximately 10-fold (for reviews see Carafoli, 1992a,b, 1994; Carafoli and Stauffer, 1994). Phosphorylation of the calmodulin-binding site by protein kinase C can affect the binding of calmodulin to that site (Hofmann et al., 1994). There is some evidence for the direct regulation of the Ca^{2+}-ATPase by hormones, such as those that mobilize Ca^{2+} (Prpic et al., 1982; Lotersztajn et al., 1984;) as well as by glucagon *in vitro* (Lotersztajn et al., 1981; Lin et al., 1983; Lotersztajn et al., 1985; see Bygrave and Benedetti, 1993 for a discussion). The possibility that this may also occur *in vivo* relies on the observation that the Ca^{2+}-ATPase can couple to a Gs-like protein (Lotersztajn et al., 1992, Jouneaux et al., 1993), this interaction being inhibitory to its function. Further recent *in vitro* studies by Takahashi and Yamaguchi (1993a,b) have indicated that Ca^{2+}-ATPase may be stimulated by regucalcin, a liver cytosolic Ca^{2+}-binding protein, as well as by a number of hormones, including calcitonin directly. This feature also endows vanadate resistance upon the molecule. The *in vivo* significance of the above observations is presently unclear. Therefore despite indications of the modulation of the Ca^{2+}-ATPase of the plasma membrane by various hormones *in vitro*, the direct stimulatory role of Ca^{2+} on the activation of the ATPase is probably the primary factor in its regulation, and therefore in maintaining intracellular Ca^{2+} homeostasis.

Whether the pump is present at the canalicular domain of the hepatocyte has not been determined with certainty. While ATPase-dependent Ca^{2+} transport has been shown in highly-enriched bile canalicular vesicles (Bachs et al., 1985, Blitzer et al., 1989), immunocytochemical data has indicated that contamination of membrane components from other regions of the membrane may have occurred (Kessler et al., 1990; Gatmaitan and Arias, 1995).

Influx of Ca^{2+} into the cell down its concentration gradient is selectively facilitated by specific Ca^{2+} channels located in the plasma membrane. The plasma membrane Ca^{2+} channels are a heterogeneous family of membrane proteins, which are multi-subunit protein complexes that form Ca^{2+} permeable pores which span the membrane. They can be functionally divided into voltage-dependent and voltage-independent groups (Felder et al., 1994). The voltage-dependent Ca^{2+} channels predominate in excitable cells

and their opening can be regulated by depolarisation of the plasma membrane (see e.g. Hosey and Lazdunsky, 1988; Hille, 1992). The voltage-independent channels are unaffected by depolarization, and their opening is associated with the stimulation of a cascade of reactions resulting from the binding of an agonist with its receptor (Fasolato et al., 1994; Felder et al., 1994; (covered in detail in Part 1, Section (III)). This latter type of channel is the predominant form in non-excitable cells, and may be exclusively present on the plasma membrane of hepatocytes (Pozzan et al, 1994). It has recently been suggested that the influx of Ca^{2+} in non-excitable cells is facilitated by hyperpolarization of the plasma membrane to more positive potentials (Clapham, 1995a) resulting from the opening of potassium channels (see also Reinhart et al., 1984b).

The mechanism by which Ca^{2+} flux occurs through Ca^{2+} channels has been derived to date from data from voltage-dependent channels. Some of these have been functionally reconstituted and expressed in *Xenopus* oocytes (McDonald et al., 1994). As a result, the relationship between the structure and function of these channels is emerging at the molecular level (Hille, 1992; McDonald et al., 1994). The kinetics of inhibition of both the voltage-dependent and voltage-independent channel types by divalent metal ions (for example Ni^{2+}) display similarities (Hughes and Barritt, 1989; Crofts and Barritt, 1990), yet there are major differences between these groups in the mechanism by which these channels are opened and the extent of their sensitivity to the organic Ca^{2+} channel inhibitors. Thus it is possible that there are similarities in the structure of the pore region, at least at the recognition site, in both these categories of Ca^{2+} channels.

Various models have been proposed concerning the mechanism of translocation of the ion through Ca^{2+} channels (see e.g Hess and Tsien, 1984; Armstrong and Neyton, 1991). In the open state, the size and affinity of the recognition site for Ca^{2+} must exclude other ions, at the same time enabling movement of the ion through the channel (Yellen, 1993). Site-directed mutagenesis studies on an L-type (dihydropyridine-sensitive) Ca^{2+}-channel protein have shown that the selectivity and flux-rate is dependent on an asymmetrical arrangement of carboxylates derived from intra-channel glutamate residues (Yang et al., 1993). Putative phosphorylation sites are present on more than one of the subunits

of the channel proteins studied so far (Hille, 1992; Castellano and Perez-Reyes, 1994; Norman and Leach, 1994). Their significance in the regulation of channel activity still requires clarification.

The extent to which the mechanism of Ca^{2+} translocation through the voltage-independent channels are similar to those described above for the voltage-dependent channels can only be ascertained when further details of the structure of these channels becomes available.

Endoplasmic Reticulum

The endoplasmic reticulum is a single, highly anastomosing organelle, which forms a single intracellular compartment (see e.g. Pozzan et al., 1994). Its important role in Ca^{2+} homeostasis results from the substantial amount of Ca^{2+} which can be sequestered within this organelle (see e.g. Taylor, 1995, and references therein; Bygrave and Benedetti, 1995) as well as the presence of mechanisms for not only rapid sequestration of the ion, but also its release into the cytosol.

Ca^{2+} is actively sequestered by the action of Ca^{2+}-ATPases located on the membrane of the organelle. These Ca^{2+}-ATPases are structurally and functionally similar to the sarcoplasmic reticular membrane Ca^{2+}-ATPases, and therefore are collectively referred to as the SERCA (Sarco-Endoplasmic Reticulum Ca^{2+}-ATPases). They also are clearly distinguishable from the plasma membrane Ca^{2+}-ATPases by virtue of their inhibition by thapsigargin (Thastrup et al., 1989, 1990), 2,5-di-*tert*-butyl-hydroquinone (BHQ) (Moore et al, 1990) and cyclopiazonic acid (Demaurex et al., 1992; Missiaen et al., 1992a), these compounds having negligible effect on the plasma membrane Ca^{2+}-ATPases (see e.g. Kass et al., 1989; Moore et al., 1990). A further difference is that the SERCA transport two Ca^{2+} ions per ATP molecule consumed, while the plasma membrane ATPases transport a single Ca^{2+} ion per ATP.

Because the endoplasmic reticular luminal Ca^{2+} concentration is considerably higher than that of the cytosol (Pozzan et al., 1994; Taylor, 1995 and references therein; Bygrave and Benedetti, 1995), its release from the endoplasmic reticulum is dependent on the regulated opening of a channel. The most important regulator of this Ca^{2+} release is the second messenger inositol 1,4,5

trisphosphate (IP_3), the generation of which is described in the next section. IP_3 interacts with a specific receptor at the endoplasmic reticulum membrane, which is associated with a Ca^{2+} channel (see e.g. Mauger et al., 1994; Pozzan et al., 1994; Taylor and Traynor, 1995). This ligand-gated Ca^{2+}-channel is a homotetramer of which each subunit is 300 kDa (Furuichi et al., 1989, Pozzan et al., 1994), whose opening probability presumably increases as a result of a conformational change in the protein mediated by the binding of IP_3. It is currently unclear whether all of the subunits are required to bind IP_3 to give an active (open) conformation (Pozzan et al., 1994). In fact feedback inhibition of Ca^{2+} release by IP_3 has been described (Hajnoczky and Thomas, 1994). Activity of the receptor/channel is also modulated by intraluminal and cytoplasmic Ca^{2+}. While the presence of Ca^{2+}-binding sites on the intraluminal sections of the protein has not been shown, luminal Ca^{2+} can increase IP_3-induced Ca^{2+} release, indicating that an intraluminal Ca^{2+}-sensing mechanism must operate (Missiaen et al., 1992b,c; Oldershaw and Taylor, 1993; Missiaen et al., 1994; Bootman et al., 1995). The cytosolic Ca^{2+} concentration has a biphasic effect on Ca^{2+} release. Initially a rise in cytosolic Ca^{2+} concentration potentiates Ca^{2+} release (Bezprozvanny et al., 1991; Finch et al., 1991; Iino and Endo, 1992; Marshall and Taylor, 1993; Combettes et al., 1994; Missiaen et al., 1994), but above a threshold concentration of approximately 300nM it becomes inhibitory (Iino and Endo, 1992). Further control of the release of Ca^{2+} is through site-specific phosphorylation of the receptor by various protein kinases (Ferris and Snyder, 1992). Phosphorylation by protein kinase A has been shown to facilitate the IP_3-mediated release of Ca^{2+} from the endoplasmic reticulum (Burgess et al., 1991; Hajnoczky et al., 1993) while phosphorylation by protein kinase G is inhibitory to Ca^{2+} release (Komalavilas & Lincoln, 1994). However, the role and significance of other phosphorylation events such as those mediated by the CaM (Hill et al., 1988) kinases, as well as its autophosphorylation (Ferris et al., 1992) has yet to be clarified.

Mitochondria

Sequestration of Ca^{2+} into liver mitochondria occurs by a Ca^{2+} uniporter (see e.g. Gunter and Gunter, 1994). Ca^{2+} influx via the uniporter involves diffusion down an electrochemical gradient, dissipation of which results in loss of Ca^{2+}-sequestering activity (see

e.g. Kapus et al., 1991), and in fact net release of the ion (see e.g Wingrove and Gunter, 1986). Ca^{2+} can be released from the mitochondria by two efflux mechanisms, one of which is Na^+-dependent and the other not. The Na^+-dependent mechanism, which has a minor role in hepatocytes, but predominates in heart and brain tissue (Wingrove and Gunter, 1986; Dawson, 1990), involves the exchange of two Na^+ ions for a Ca^{2+} ion. The poorly-defined Na^+-independent mechanism predominates in mitochondria from hepatocytes (Wingrove and Gunter, 1986; Dawson, 1990), and probably acts through the exchange of two H^+ ions for each Ca^{2+}. Both mechanisms appear to be active Ca^{2+} transport systems, but require further characterization (Gunter and Gunter, 1994). The role of the permeability transition pore in the transport of Ca^{2+} across the mitochondrial inner membrane is also presently unclear (Gunter et al., 1994).

Other organelles

Other organelles and vesicular structures potentially have Ca^{2+}-sequestering activity, although their importance in the regulation of intracellular Ca^{2+} homeostasis is presently debatable. The nucleus has been suggested as one putative Ca^{2+}-storing organelle. The nuclear envelope is continuous with the endoplasmic reticulum membrane, and therefore contains similar structures (e.g. the IP_3 receptor) to those found on the endoplasmic reticulum. Recent work by al-Mohanna et al. (1994) has shown that while small changes in cytosolic Ca^{2+} are immediately reflected in the nucleus, large changes are attenuated, indicating a degree of insulation of the nucleus from cytosolic Ca^{2+} changes. Furthermore, modulation of nuclear Ca^{2+} concentration by changes in IP_3 levels has been shown (see e.g. Hajnoczky et al., 1994). However, in general, the extent to which changes in intracellular Ca^{2+} in this region are independent of changes in cytosolic Ca^{2+} is not presently clear. Recently, the Golgi apparatus have been shown to sequester Ca^{2+} in some cell types (Zha et al., 1995). The significance of this activity is not yet clear.

Since under basal (i.e. non-stimulated) conditions the cytosolic Ca^{2+} concentration is maintained at low concentrations within fairly narrow limits, a consequence of the constant cycling of Ca^{2+} across the membranes of Ca^{2+} sequestering organelles and the plasma

membrane, is that any stimulus which affects the rate of flux of Ca^{2+} across any of these membranes also impacts on that across the other membranes. The coupling between receptor binding and Ca^{2+} mobilization will now be discussed in detail in the next sections, as well as in the General Discussion.

(III) Overview of the mechanism of action of Ca^{2+}-mobilizing hormones

The Ca^{2+}-mobilizing hormones are those for which the mobilization of Ca^{2+} follows their interaction with a cell membrane receptor. This is a fundamental component of the mechanism by which they induce metabolic responses. In rat liver this mechanism applies to the hormones vasopressin, angiotensin II and adrenalin (via the α_1-adrenergic receptors), among other physiological agonists. As the mechanism of Ca^{2+} mobilization by these agents has been detailed in several recent reviews (see e.g. Altin and Bygrave, 1988; Putney, et al., 1989; Dawson, 1990; Irvine, 1992; Berridge, 1993; Bygrave and Benedetti, 1993; Putney and Bird, 1993a; Nathanson, 1994; Pozzan et al., 1994; Clapham, 1995a), as has the inositol lipid signalling pathway which is central to these events (Putney and Bird, 1993a; Balla and Catt, 1994; Clapham, 1995a; Divecha and Irvine, 1995), only a brief overview will be presented here.

Agonists increase the intracellular Ca^{2+} concentration either by inducing the release of Ca^{2+} from an intracellular (agonist-releasable) Ca^{2+} store such as the endoplasmic reticulum, and/or by promoting the influx of Ca^{2+} from the extracellular medium. The mechanism by which the release of Ca^{2+} from the endoplasmic reticulum occurs has been defined in some detail, but the mechanism of influx from the extracellular medium has been more elusive. The co-ordination of these events, as well as their temporal and functional relationship is gradually emerging (see General Discussion).

The interaction of the agonist with its receptor triggers a G-protein-mediated stimulation of the intracellular enzyme phospholipase C. The activated phospholipase C catalyses the dissociation of phosphatidylinositol (4,5) -diphosphate (PIP_2) into inositol(1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) (see reviews above). The relative biological significance of other

derivatives of *myo*-inositol which are present in mammalian cells is gradually emerging (see e.g. Toker et al., 1994). In addition synthetic variants of IP_3 and IP_4 have been produced with potent biological activities (Wilcox et al., 1994).

The action of a second messenger on the channel has been suggested as a mechanism by which the stimulation of Ca^{2+} influx occurs, and this role has been ascribed to IP_3 (Striggow and Bohnensack, 1994) as well as its immediate metabolite, IP_4 (see e.g. Parker and Ivorra, 1991; Lückhoff and Clapham, 1992; Shirakawa and Miyazaki, 1995; but see Striggow and Bohnensack, 1994). The presence of IP_3 receptors on the plasma membrane of various cell types has been shown (see e.g. Kuno and Gardner, 1987; Fujimoto et al., 1992). An IP_3 receptor isolated from the plasma membrane of liver cells has been characterized (Mayrleitner et al., 1995) which, in common with the IP_3 receptor found on the endoplasmic reticular membrane, has Ca^{2+}-channel activity, but which is also distinguishable from it by a number of unique properties (see Mayrleitner et al., 1995). It remains to be established whether this is a subtype of the IP_3 receptor which is localized on regions of the endoplasmic reticulum that are closely associated with the plasma membrane and which co-purify with the plasma membranes (Lievremont et al., 1994). Whether all of this putative plasma membrane IP_3 receptor is derived from the plasma membrane of hepatocytes or from other cell types in the liver also requires clarification.

A direct G-protein-mediated interaction between the receptor and the Ca^{2+} channel could be expected to be quite rapid, due to the localization of both structures on the plasma membrane. Thus any G-protein-mediated influx of Ca^{2+} should precede any Ca^{2+} release from intracellular stores (see general discussion). In fact, this has been shown to be the case under certain conditions with human platelets (Sage and Rink, 1987), parotid acinar cells (Merritt and Rink, 1987) and GH_4C_1 cells (Tashjian et al., 1987), but despite preliminary reports indicating that this effect occurs in hepatocytes (Blackmore, 1988; Exton, 1988), this has not been confirmed.

A depletion-operated mechanism for Ca^{2+}-channel activation would indicate a more indirect action for IP_3. That is, the depletion of the Ca^{2+} content of the endoplasmic reticulum by IP_3 would

result in an influx-stimulating signal being transmitted to the plasma membrane. By depleting the endoplasmic reticular Ca^{2+} stores without IP_3, with agents such as thapsigargin, cyclopiazonic acid or 2,5-di-*tert*-butyl-hydroquinone (BHQ), which inhibit the Ca^{2+}-ATPase of the endoplasmic reticulum membrane (see e.g. Thastrup et al., 1989), it has been established that influx of Ca^{2+} can indeed be induced in this way (Takemura et al., 1989; Glennon et al., 1992). In this situation a retrograde signal needs to be produced which communicates the Ca^{2+} status of the endoplasmic reticulum to the Ca^{2+} channels at the plasma membrane, inducing influx of the ion. A Ca^{2+}-influx factor (CIF) has been isolated from some cell types (Randriamampita and Tsien, 1993; Parekh et al., 1993) and characterization of this factor is in progress (Radriamampita and Tsien, 1995; but see Bird et al., 1995; Gilon et al., 1995). The extent to which a factor might be involved intracellularly in the stimulation of influx induced by Ca^{2+}-mobilizing hormones remains to be established (see e.g. Clapham, 1995b). Putative factors suggested include cytochrome P450 (Alvarez et al., 1992) and small GTP-binding proteins (see e.g. Putney and Bird, 1993b; Fasolato et al., 1993). It has further been suggested that cyclic GMP or nitric oxide might have a role in these events (see e.g. Xu et al., 1994; Milbourne and Bygrave, 1995), and work is underway to uncover the role of this agent.

In summary, three putative mechanisms have been proposed by which the voltage-independent Ca^{2+} channels might be activated by the binding of a Ca^{2+}-mobilizing agonist to its receptor to induce influx of Ca^{2+}; evidence exists for all three types (see Fasolato et al., 1994; Felder et al., 1994 for recent reviews). These are: (1) Influx by the second messenger-operated Ca^{2+} channels can be induced by a second messenger generated as a result of the interaction between an agonist and its receptor (see any of the reviews listed earlier). (2) The interaction between a Ca^{2+}-mobilizing agonist and its receptor can result in a direct (Irvine, 1990; Kraus-Friedmann, 1994), or G-protein-mediated stimulation of influx of Ca^{2+} through the receptor-operated Ca^{2+}-channels without the requirement for a second messenger (Blackmore, 1988; Exton, 1988). (3) The depletion-operated Ca^{2+} channels respond to depletion of the intracellular Ca^{2+} stores, which results in the generation of a retrograde messenger molecule, which in turn stimulates the opening of the Ca^{2+} influx channel.

The mechanisms for Ca^{2+} influx discussed above are not mutually exclusive; two or more of these mechanisms may operate at any one time. For example, evidence has been provided for the influx induced by vasopressin and thapsigargin occurring via the same pathway (Strazzari and Hughes, 1993) as well as for the presence of separate IP_3-mediated and depletion-mediated pathways in hepatocytes (Llopis et al., 1992; Striggow and Bohnensack, 1994). Additionally it has been shown that store-depletion mediated Ca^{2+}-inflow requires the pertussis-toxin sensitive G_{i2}-protein (Berven and Barritt, 1994; Fernando and Barritt, 1994a). The extent to which each of these mechanisms operates at any given time may be especially relevant to the mechanisms by which differences in the Ca^{2+}-flux pattern are induced with different concentrations of the agonists (see General Discussion), as well as to situations where cross-talk between the signalling pathways is occurring (Bygrave and Benedetti, 1993) This will be discussed in the next sections (see Part I, Section (D) (V) of this review).

(IV) The cyclic AMP signalling pathway

The interaction of glucagon with its receptor in hepatocytes and the resultant generation of cyclic adenosine monophosphate (cyclic AMP) as a second messenger, is of considerable physiological significance. One result, for example, is the stimulation of the glycogenolytic cascade, thereby contributing to the control of blood glucose concentration. In view of the potential cellular and physiological significance of the modulation by the cyclic AMP signalling pathway of the responses induced by the Ca^{2+}-mobilizing agonists, a brief overview of the mechanism by which cyclic AMP is produced, as well as the mechanism by which it regulates the activity of protein kinase A (PKA) is now presented.

The interaction of glucagon with its receptor results in a G-protein (G_s)-mediated stimulation of the membrane-bound enzyme adenylate cyclase, which catalyses the formation of cyclic AMP from adenosine triphosphate (ATP). Most of the cellular responses of cyclic AMP are mediated by its stimulation of PKA activity (reviewed in e.g. Taylor et al., 1990; Barritt, 1992; Francis and Corbin, 1994a).

In the non-activated state, PKA is present as a dimer, with each monomer comprising a regulatory subunit as well as a catalytic subunit. The binding of cyclic AMP to a specific site on the regulatory subunit results in the release of the catalytic subunit from the complex. This event is accompanied by a positive cooperativity for the binding of cyclic AMP to another site on the molecule (Barritt, 1992; Francis and Corbin, 1994a). The activity of various intracellular proteins is regulated by their phosphorylation at specific sites by the catalytic subunit. In this way the stimulation of the cyclic AMP cascade by glucagon has a central role in cellular regulation.

The regulation of the production as well as degradation of cyclic AMP adds further control on cyclic AMP-mediated events. The G_i G-protein inhibits the activity of the adenylate cyclase. The interaction has been shown recently to be facilitated as a result of a protein kinase C-mediated phosphorylation of the α-subunit of the inhibitory G-protein G_i-2 (Morris et al., 1994; Savage et al., 1995). As protein kinase C activity is stimulated in response to stimulation by the Ca^{2+}-mobilizing agonists, the latter may play a significant role in the regulation of cyclic AMP concentrations. In addition, significant protein kinase C activity is induced by glucagon, resulting from the stimulation of phosphatidylcholine metabolism (Pittner and Fain, 1991) as well as phosphatidylinositol metabolism (Tang and Houslay, 1992; for a review see Bygrave and Roberts, 1995), indicating a self-regulatory mechanism for this agonist. Further interactions with $\beta\gamma$-subunits of G-proteins have been found to be inhibitory with some adenylate cyclase subtypes (AC-1) and stimulatory or ineffective in others (Chen et al., 1995).

A further aspect of the role of cyclic AMP in cellular regulation is the possible role of negative feedback by cyclic AMP. For example, the cyclic AMP phosphodiesterases are known to be stimulated by PKA-mediated phosphorylation (see e.g. Corbin et al., 1985; Gettys et al., 1987; Barritt, 1992). In the liver, both cytosolic and plasma membrane-associated forms of this enzyme exist, at least one variety of the latter type being activated by Ca^{2+} (Barritt, 1992). This may partially explain the stimulation of phosphodiesterase activity by vasopressin in this cell type (Keppens and De Wulf, 1984; Miot et al., 1988).

It has been suggested that cyclic AMP may mediate effects which are distinct from those mediated by PKA activation. While examples of these could include independent effects of the cyclic AMP regulatory subunit complex, stimulation of another as yet undiscovered cyclic AMP-binding protein or cross-activation with cyclic GMP-binding sites (for discussion see Francis and Corbin, 1994a,b), experimental clarification is still forthcoming.

(V) Cross-talk between the cyclic AMP/ phosphoinositide signalling pathways

Cross-talk between the cyclic AMP and phosphoinositide signalling pathways is considered to be important in the modulation of a number of cellular functions, including the flow of bile (Bygrave and Benedetti, 1993; Bygrave et al., 1994). Central to the mechanism by which this is achieved is the synergistic stimulation of Ca^{2+} influx which occurs as a result of this interaction (extensively reviewed in Bygrave and Benedetti, 1993). This synergistic response has been shown to occur in hepatocytes (Mauger et al., 1985; Combettes et al., 1986, Burgess et al., 1986, 1991, Kass et al., 1990) as well as in the perfused rat liver (Altin and Bygrave, 1986, 1988; Chapter 1 and 3, this work). The latter studies have shown that one important feature of this synergism is an enhancement of both the rate and duration of the influx, resulting in a large net uptake of the ion. In the presence of glucagon, the time between the administration of the Ca^{2+} agonist and the response is considerably decreased (see e.g. Chapter 1), an effect which can also be observed to some extent by increasing the concentration of the agents (Chiavaroli et al., 1994; Chapter 3, this work). One physiological role for cross-talk in this context therefore may be to sensitise the system to enable physiological concentrations of hormones to stimulate metabolic events. The loci on the pathways at which this cross-talk might occur to promote the influx of Ca^{2+} still currently requires clarification. However a number of clues have emerged, and these will now be reviewed.

The role of emptying of intracellular Ca^{2+} stores in the stimulation of influx of the ion was alluded to in a previous section (see Section (III)). Pertinent to this discussion is the observation that glucagon is able to sensitize intracellular Ca^{2+} stores for Ca^{2+} release (Burgess et al., 1986, 1991; Mauger et al., 1989; Hajnoczky

et al., 1993), as reflected in both the amount of Ca^{2+} released and a lower EC_{50} for Ca^{2+} release (Bygrave and Benedetti, 1993). This effect has been traced to a protein kinase A-mediated phosphorylation event at the IP_3 receptor (Burgess et al., 1991; Hajnocky et al., 1993). Thus the mechanism by which the greater influx is stimulated could potentially be related to an increased efficiency of Ca^{2+} release from the intracellular stores (see General Discussion). Supporting this is the observation that vasopressin-induced influx of Ca^{2+} has been demonstrated to occur following the interaction between agents which deplete the endoplasmic reticular Ca^{2+} pool (such as BHQ and thapsigargin) and vasopressin (Kass et al., 1990, 1994a, General Discussion). Therefore a synergistic interaction with multiple factors may be involved in maximally stimulating the influx rate. Whether the evidence for the involvement of multiple Ca^{2+} transport systems in Ca^{2+} influx (Barritt et al., 1981; Hughes et al., 1986; Altin and Bygrave, 1987; Barritt and Hughes, 1991; Llopis et al., 1992; Kass et al., 1994b; General Discussion), or the existence of IP_3-sensitive and -insensitive Ca^{2+} stores (Gamberucci et al., 1995) are relevant to this question, remains to be determined.

An early suggestion that influx results from an increased ability of intracellular stores, such as the mitochondria, to take up the ion (Prpic et al., 1978; Morgan et al., 1983; Altin and Bygrave, 1986; Barritt and Hughes, 1991; General Discussion) remains a possibility. Further dual mechanism models exist, including the possibility of an interaction by the second messengers at a plasma membrane Ca^{2+} channel as well as at the IP_3 receptor channel (see e.g. Striggow and Bohnensack, 1994), but clearly further clarification is required.

Since the release of Ca^{2+} from the intracellular stores can sensitise Ca^{2+} influx, and since glucagon can induce the release of Ca^{2+} (reviewed in Bygrave and Benedetti, 1993), it may be argued that the synergistic influx induced by glucagon and vasopressin, results from prior depletion of intracellular Ca^{2+} stores by glucagon. Thus it is appropriate to examine the mechanism by which Ca^{2+} mobilization by glucagon occurs.

Glucagon appears able to stimulate a small amount of IP_3 production (Wakelam et al., 1986; Mine et al., 1993; Tang and

Houslay, 1992; Bygrave and Roberts, 1995), which however is more likely to be attributable to events at the level of the glucagon receptor rather than to cyclic AMP (Staddon and Hansford, 1988; Bygrave and Roberts, 1995). The observations that influx of Ca^{2+} is considerably potentiated even at concentrations much lower than those which induce any release of Ca^{2+} from the perfused liver (see Chapter 3), as well as when glucagon is added after the Ca^{2+}-mobilizing agonists (Altin and Bygrave, 1986), argue against the significance of a role for prior depletion of intracellular stores by glucagon in the stimulation of influx. However, whether the observation that glucagon also is able to stimulate phosphatidylcholine metabolism (Pittner and Fain, 1991) is of any significance to the phenomena described (Bygrave and Benedetti, 1993) remains to be determined.

The potentiation of Ca^{2+} influx by cyclic AMP may occur at the receptor level. Analogous to the differences between responses in Ca^{2+} fluxes induced by stimulation of the α-adrenergic receptors and vasopressin receptors (Dasso and Taylor, 1994), facilitation of a receptor-G-protein interaction by cyclic AMP has been suggested (Bygrave and Roberts, 1995). Indeed, protein kinase A has been shown to phosphorylate both the vasopressin (Pittner and Fain, 1989) and α_1-adrenergic (Bouvier et al., 1987) receptors. While the full significance of this phosphorylation is not clear, it raises the possibility that an interaction between the receptor and its associated G-proteins is facilitated.

Cyclic AMP could enhance IP_3 production which results from the action of Ca^{2+}-mobilizing agonists. Variable data have been presented on the effect of cyclic AMP on the production of IP_3, a potentiation of its production being observed in some studies (Blackmore and Exton, 1986; Whipps et al., 1987; Pittner and Fain, 1989a, 1990), with no change in others (see Bygrave and Benedetti, 1993 for discussion). In those studies where increased IP_3 has been observed in the presence of cyclic AMP, it does not appear to be sufficiently large by itself to explain the extent to which Ca^{2+} influx is modulated. Similarly some evidence indicates that the combination of glucagon and vasopressin induces a higher cytosolic Ca^{2+} concentration than each hormone acting alone (Combettes et al., 1986; Joseph and Williamson, 1986). The magnitude of this increase is considerably smaller than the extent to which the rate of

influx is enhanced in the perfused liver system, indicating that the Ca^{2+} taken up is efficiently cleared into intracellular storage sites.

The Ca^{2+} responses tend to decay even during continued stimulation with the hormones, and this may involve receptor internalization (see e.g. Nathanson et al., 1994) as well as the operation of feedback mechanisms on components of the signalling pathways, for example on phospholipase C. They may result also from the filling of intracellular Ca^{2+} stores.

The mechanism by which Ca^{2+} is exchanged between the storage sites and the cytosol, and the spatio-temporal aspects of Ca^{2+} movement across the cytosol will be the covered in the following section.

(VI) Spatio-temporal aspects of Ca^{2+} signalling in hepatocytes

Intracellular Ca^{2+} has a short half-life, largely due to a combination of the affinity of the ion for the abundant Ca^{2+}-binding components in the cell and the activity of the Ca^{2+}-ATPases. This results in a limited diffusion distance for each Ca^{2+} ion (see e.g. Kasai and Petersen, 1994), and a non-uniform distribution of the ion within the cell. For example, cycling of Ca^{2+} across membranes would presumably result in a higher submembrane Ca^{2+} concentration, with consequences for any Ca^{2+}-sensitive proteins located at the membrane (for reviews see e.g. Bygrave, 1978; Rasmussen, 1989). Therefore it has been of interest in recent years to examine changes in Ca^{2+} over time at various points in the cell in detail, along with any physiological implications.

Scrutiny of the changes in intracellular Ca^{2+} concentration at high resolution has been made possible by advances in microscopic imaging technology (see e.g. Nathanson, 1994, for a review). At this increased resolution the agonist-induced increases in intracellular Ca^{2+} concentration consist of a series of oscillations (Woods, et al., 1986; see e.g. Petersen et al., 1994 for a review). These global oscillations observed at a point in the cytosol actually translate into waves which move across the cell, having a distinct initiation site (pacemaker) and direction. Therefore stimulation of a cell by an agonist results in localised pockets of high Ca^{2+} concentrations of short duration and range (Petersen et al., 1994).

The magnitude and duration of a Ca^{2+} wave at a point in the cell would be determined by the distribution and characteristics of the Ca^{2+} stores, as well as the status of entry of extracellular Ca^{2+}. This spatio-temporal aspect of Ca^{2+} signalling will be discussed below.

The stores involved with the oscillation of Ca^{2+} are those which are able to rapidly release as well as sequester significant amounts of the ion. Consequently the endoplasmic reticulum and the mitochondria are implicated.

The properties (frequency and shape) of Ca^{2+} oscillations are characteristic of the type of agonist used (Rooney et al., 1989; Thomas et al., 1991; Sanchez-Bueno and Cobbold, 1993), as well as agonist concentration (Woods et al., 1986; Rooney et al., 1989) and other experimental conditions (Renard-Rooney et al., 1995). There is also cell type specificity in the oscillations produced (Nathanson, 1994), and they can even be influenced by the state of differentiation of the cell (Kitamura et al., 1995). At moderate concentrations of agonist, the oscillations tend to be transient, returning to basal levels between peaks. At high concentrations of some agonists, the oscillations in intracellular Ca^{2+} concentration occur around an elevated baseline, (sinusoidal oscillations) in some cases being replaced by a sustained increase in intracellular Ca^{2+} (Putney and Bird, 1993a; Petersen et al., 1994).

The mechanism by which these oscillations are generated and propagated as waves is not presently clear. The cycle of release of Ca^{2+} from the endoplasmic reticulum probably involves some of the feedback and stimulation parameters discussed in Part 1, Section (II). Since evidence has been proposed for IP_3 receptors being heterogeneous in their affinity for IP_3, it has been suggested that the initiation of the oscillation could involve a region of the endoplasmic reticulum where high-affinity IP_3 receptors exist, while the propagation of the wave of Ca^{2+} would involve low-affinity IP_3 receptors (D'Andrea et al., 1994). By this model a complex stimulation-feedback cycle would operate within the time-frame of each spiking event (Petersen and Berridge, 1995). Various models have been proposed to mathematically describe the mechanism behind the cytosolic Ca^{2+} oscillations (see e.g. Berridge and Gallione, 1988; Meyer and Stryer, 1988, 1991; Swillens and Mercan, 1990; Keizer and de Young, 1992; Røtnes and Røttingen,

1994; Stucki and Somogyi, 1994). These generally centre on the interplay of positive cooperativity and negative feedback of Ca^{2+} and IP_3 at the IP_3 receptor (see e.g. Petersen et al., 1994; Nathanson, 1994). A further point of control could be at the level of IP_3 synthesis. Although IP_3 generation has been suggested to follow an oscillatory pattern (see e.g. Stucki and Somogyi, 1994), it probably is not the primary mechanism by which the oscillations in intracellular Ca^{2+} are generated since Ca^{2+} oscillations occur even where the IP_3 level has been kept constant (Wakui et al., 1989, Thorn, 1993). The role of mitochondria and extracellular Ca^{2+} in these events is less clear. While the mitochondria have generally been thought to have a passive role in these waves of Ca^{2+}, recent analysis of the kinetics of the Ca^{2+} fluxes indicate that Ca^{2+} uptake into the mitochondria can occur even at concentrations of Ca^{2+} of 200-500nM (Gunter and Gunter, 1994; Sparagna et al., 1994). At very short times (less than 1s) the uniporter appears to be in a conductivity state where the uptake of Ca^{2+} is very fast, suggesting that uptake of the ion during short pulses would be possible (Gunter and Gunter, 1994). Further, it also has been shown that even small increases in cytosolic Ca^{2+} resulting from IP_3-induced release of the ion from the endoplasmic reticulum, can be taken up by mitochondria (Rizzutto et al., 1993). In fact it has recently been shown that the respiratory status of mitochondria can modulate the activity of Ca2+ waves (Jouaville et al., 1995; General Discussion) Unless release of Ca^{2+} from the mitochondria also occurs during oscillations a gradual redistribution of the ion could occur during agonist stimulation. While studies with the perfused liver have shown that Ca^{2+} is the only second messenger which has a direct effect on mitochondria (Denton and McCormack, 1985; McCormack and Denton, 1993, 1994; Gunter et al., 1994), the influence of other signals on Ca^{2+} movements into and out of the mitochondria cannot be ruled out. Candidates for agents which appear to promote Ca^{2+} influx into the mitochondria include protein kinase A (see Altin and Bygrave, 1988) as well as (inositol hexakisphosphate (IP_6) (Copani et al., 1991) and cyclic GMP.

Since the SERCA inhibitor thapsigargin has been shown to induce oscillations of Ca^{2+} (Burgess et al., 1986, 1991), under these conditions the pools to which the Ca^{2+} can be removed following a spike of Ca^{2+}, are limited to the mitochondria or extracellular medium. In fact hormone-induced net influx of Ca^{2+} is actually

considerably enhanced in the presence of SERCA inhibitors. Therefore in these situations the destination of Ca^{2+} must also be the mitochondria. Similarly, mitochondria are able to sequester considerable amounts of Ca^{2+} where influx has been induced by cross-talk between the agonists, although the extent of Ca^{2+} sequestration varies according to the methodology used to obtain the data (see e.g. Altin & Bygrave, 1986, 1988; Benedetti et al., 1989; but see also Bond et al., 1987).

The extent to which extracellular Ca^{2+} is involved, and the mechanisms by which these rises in Ca^{2+} can be generated and inhibited to form distinct peaks within a short time span, as well as their co-ordinated propagation across the cell, require further investigation. In this respect it is noteworthy that cross-talk resulting from the interaction between the cyclic AMP and phosphoinositide signalling pathways is also reflected in Ca^{2+} oscillations (Sanchez-Bueno et al., 1993). Minimal spiking was induced by glucagon or cyclic AMP alone, and only at high concentrations, consistent with data from the perfused rat liver, where these agents have been shown to induce only a small efflux of Ca^{2+}. However glucagon or cyclic AMP was able to modify the spiking pattern induced by vasopressin or phenylephrine, resulting in a substantial increase in the frequency of this event, as well as a slight increase in its amplitude (Sanchez-Bueno et al., 1993). Whether the oscillations of extracellular Ca^{2+} observed with the perfused liver under certain circumstances are related to the oscillations observed in hepatocytes, remains to be determined. The mechanism by which the phase of Ca^{2+} oscillations is coordinated when multiple pools are involved in their generation and propagation is an intriguing question.

Agonist stimulation therefore probably results in the transient formation of localised regions with high Ca^{2+} concentrations. The importance of cellular microdomains in cellular function is presently unclear, but as pointed out in the reviews of Cheek (1989) and Nathanson (1994), a consequence of this would be the facilitation of Ca^{2+}-sensitive functions at their cellular locations, such as the stimulation of secretory events at the apical pole of secretory cells. A corollary of the above is that inappropriate cellular localization of pockets of either high or low Ca^{2+} concentration may have a negative effect on cellular functions.

Thus the physical location and also the mobility of structures which are able to influence the cytosolic Ca^{2+} concentration, whether organelles or endogenous Ca^{2+} buffers, can determine cellular function. For example vesicles derived from the endoplasmic reticulum which have Ca^{2+} storing, releasing and sequestering capability, may be able to release that Ca^{2+} in response to agonist stimulation (Petersen et al., 1994). In view of the fact that an intracellular signal(s) is(are) probably generated in response to Ca^{2+} depletion within the endoplasmic reticulum resulting in the influx of Ca^{2+}, the intriguing possibility arises that a further interaction of this signal with components of the signalling pathways described earlier may itself affect Ca^{2+} fluxes and/or cellular function. A further dimension is added by the suggestion that the oscillations in themselves may encode cellular signals, as has been shown in some cell types (Leong and Thorner, 1991; Schulman et al, 1992). In fact as is well known, a major secretory event of the liver is the production and release of bile, and the role of Ca^{2+} in the regulation of bile secretion will form Part II of this review.

INTRODUCTORY REVIEW

PART 2

Bile flow and its regulation by signalling cross-talk in rat liver

(E) Bile flow and its regulation by signalling cross-talk in rat liver

The literature on many aspects of bile flow is extensive, and a detailed review is beyond the scope of this work. I have chosen to concentrate this discussion on the role of hormones and Ca^{2+} on the regulation of bile flow. Accordingly, only a brief outline of the fundamentals of bile flow will be presented. However, the reader is referred to a number of recent reviews on various aspects of the subject below.

The synthesis of bile acids:
Vlahcevic et al., 1991; Russell and Setchell, 1992.
The uptake of bile acids into the hepatocytes:
Roda et al., 1993.
The transcellular transport of bile acids:
Stolz et al., 1989; Erlinger, 1990, 1993; Suchy, 1993.
Canalicular secretion of bile acids:
Meier, 1993; Hoffman, 1989b.
The role of non-bile acid ion transport in bile flow:
Boyer et al., 1992; Fernandez-Checa et al., 1993.
Vesicle transport:
Burgoyne and Morgan, 1993; LeSage, 1993;
Marks and LaRusso, 1993; Nuoffer and Balch, 1994;
Marks et al., 1993.
The microfilamentous network:
Phillips et al., 1983; Janmey, 1994.
The tight junctions:
Hardison, 1993; Reichen, 1993a.

(I) Introduction

Bile is the exocrine secretion of the liver, and consists of a complex aqueous solution of organic as well as inorganic components (Erlinger, 1982). It is the route for excretion from the body of potentially toxic endogenous or exogenous compounds and their metabolites (Coleman, 1987). The secretion and transportation of bile acids to the intestinal tract is important for the emulsification and consequent absorption of fats derived from the diet (see e.g. Coleman, 1987, Hoffman, 1989). Therefore optimization of the secretory as well as excretory functions of bile

requires that the flow of bile is maintained. The mechanisms involved in the flow of bile are sparsely known. Similarly, intrahepatic mechanisms involved in the perturbation of the flow of bile resulting in the clinical syndrome known as cholestasis have only been partially elucidated (for recent reviews see, e.g. Nathanson and Boyer, 1991; Reichen 1993b). In many secretory cell types the requirement for extracellular Ca^{2+} and hormone action for secretion has been shown (Douglas and Poisner, 1963; Roberts, 1990; Nauntofte, 1992; Petersen, 1992; Toescu, 1995). The mobilization of Ca^{2+} (Putney, 1977) and the generation of cyclic AMP (see Young et al., 1987 for a review) generally stimulate secretion. In fact, the interaction (cross-talk) between these pathways results in potentiation of secretion (Gardner and Jackson, 1977; Yoshimura and Nezu, 1992; Tsunoda, 1993), concomitant with which is a synergistic stimulation of the influx of Ca^{2+} (see Part I, Section (D) (V)). The importance of Ca^{2+} in regulated exocytosis has been shown in various tissues (see e.g. Burgoyne and Morgan, 1993, for a review). The role of phosphorylation of proteins on the excretory vesicle and/or the membrane acceptor site is probably significant. This work will review the literature concerning the mechanisms involved in bile flow, concentrating especially on the role of Ca^{2+} and hormones which influence Ca^{2+} homeostasis in these events.

The driving force for bile flow is thought to be the generation of an osmotic gradient across the canalicular membrane by the active secretion of relatively impermeant solutes into the canaliculus (see e.g. Sperber, 1959; Ballatori and Truong, 1992). As a consequence of the accumulation of these solutes, the movement of water into the canalicular lumen is promoted through pores in the plasma membrane (Boyer et al., 1992), but also paracellularly through the tight junctions (Graf, 1990; Ballatori and Truong, 1992). Thus compounds which affect bile flow are described as having a particular choleretic potential (expressed as µl per µmole). While this hypothesis is currently considered to provide the most tenable model for the secretion of bile, it is however not all encompassing (see e.g. Lenzen et al., 1993 for discussion), and the involvement of other factors cannot be completely discounted. The above suggests a direct link between the activity of the transport systems involved in the secretion of these solutes and the flow of bile. The bile acids are considered to be the most important organic anions involved in

forming this gradient, their rate of secretion into the canaliculus being directly related to the bile flow rate (Wheeler and Ramos, 1960; Preisig et al., 1962). Their enterohepatic circulation will be considered below.

Thus, in the enterohepatic circulation of the bile acids they are secreted by the hepatocytes into the canaliculus, from where they travel along the biliary tree via the gall bladder to the duodenum. Following their absorption into the blood from the gastrointestinal tract, the bulk of which occurs at the terminal ileum, they are presented to the liver via the portal circulation, where they are taken up into the hepatocytes. This process ensures their efficient utilisation, the most important factor in this being the processes which result in their uptake by the liver (up to 90% first-pass fractional extraction (Hoffman, 1989a)) from the blood. Since the structure of a bile acid largely determines the extent of its uptake by carriers on the hepatocyte plasma membrane (Bellentani et al., 1987; Hardison et al., 1991; Nishida et al., 1995), variations between the enterohepatic circulation of different bile acids is observed (Hoffman, 1989a).

The high degree of efficiency with which this enterohepatic circulation operates is especially attributable to these efficient mechanisms for the uptake of the bile acids located on the hepatocytes, but also on the absorption mechanisms present on intestinal cells (Dietschy, 1968). The enterocytes which line the terminal ileum employ an active Na^+-coupled co-transport system for the uptake of bile acids which is similar to that of hepatocytes (Dietschy, 1968; Hoffman, 1989). Following their transcellular passage through the enterocyte, their transport into blood probably occurs via an ion-exchange system (Weinberg et al., 1986). Biotransformations of the bile acids occur at two sites during their passage within the enterohepatic circulation: (1) The major biotransformations induced by the hepatic enzymes are amidations, which result in the conjugation of the bile acids with glycine and taurine and (2) deconjugations and dehydrogenations are performed by intestinal bacteria (Hoffman, 1989a).

The role of the gallbladder and ductular cells in modulating the flow of bile as well as its composition is acknowledged, but are beyond the scope of this review, which will focus on the role of

hepatocytes, and therefore primary canalicular events involved in the flow of bile.

The role of other endogenous organic solutes with a high choleretic potential, such as glutathione (Ballatori and Truong, 1989, 1990, 1992) probably contribute to the fraction of bile flow described as bile acid-independent.

(II) Role of Ca^{2+} and hormones in the regulation of bile flow

(a) Influence of extracellular Ca^{2+}

Studies focusing on the role of Ca^{2+} in bile flow have been prompted in part by the observation that bile flow would cease when the extracellular free Ca^{2+} concentration is decreased below approximately 100μM (Graf, 1975; Owen, 1977; Reichen et al., 1985). This cholestasis has largely been attributed to an increased tight-junctional permeability, as a result of which bile is able to leak out of the canalicular space into the sinusoidal space. Therefore it is appropriate to examine the role of Ca^{2+} in junctional integrity. It is well known that removal of Ca^{2+} from the perfusion medium results in a disruption of the adhesion between cells, and this is utilized in most methods for the isolation of hepatocytes (see e.g. Berry and Friend, 1969). The tight junctions comprise a number of peripherally-associated membrane proteins, the most characterized of which is the ZO-1 protein. Evidence from studies in other epithelial cells has indicated that Ca^{2+}-free incubation causes loss of ZO-1 from the membrane, and this is reversed upon Ca^{2+} readdition (Siliciano and Goodenough, 1988); calmodulin is involved in this event (Balda et al., 1991). Conflicting data have been presented on the role of protein kinase C in the assembly and dissassembly of junctional proteins (Balda et al., 1992).

Ultrastructural work has indicated that hepatocyte tight junctions are not disrupted during a hypocalcaemic perfusion (Drochmans et al., 1975; Miyai & Hardison, 1982; Stammler et al., 1990; Hardison, 1993). In addition it has been found that incubating hepatocyte canalicular membrane preparations in Ca^{2+}-free medium fails to disrupt tight junctions (Stevenson & Goodenough, 1984). Thus a disruption of the tight junction may not be the primary mechanism for inducing the cholestasis seen in

hypocalcaemic conditions. A substantial peri-tight junctional submembrane Ca^{2+} concentration is crucial for junctional integrity, although the site of action is presently not clear. The possibility arises that the Ca^{2+} deprivation-induced cholestasis could be related to a perturbation of plasma membrane Ca^{2+} cycling induced by the decreased extracellular Ca^{2+} concentration, although this could well be coupled with a role for extracellular Ca^{2+} in stabilizing lipids and proteins on the membrane surface. Stammler et al. (1990) found a disturbance of osmotic equilibrium in calcium deprivation-induced cholestasis, possibly involving a K^+ transport system, suggesting the involvement of mechanisms involved in cell volume regulation.

(b) Ca^{2+}-mobilization and bile flow

The possibility that the effect of Ca^{2+}-depletion on the flow of bile could be due to a perturbation in intracellular Ca^{2+} homeostasis is further suggested by the observations that agents which mobilize Ca^{2+} also induce changes in bile flow. Addition of a Ca^{2+}-mobilizing agonist to the perfused rat liver evokes within seconds a highly reproducible, transient increase in the bile flow rate, which peaks at 30-50% above the basal rate (see Fig.1, Hamada et al., 1992a; Nathanson et al., 1992a; Bygrave et al., 1994). A similar response can be observed following administration of exogenous ATP and phenylephrine (Graf, 1975; Krell et al., 1985). These effects have been associated with an increased secretory pressure in couplets (Nathanson et al., 1992b). Within 3 minutes the bile flow has decreased to about 30% of the original basal rate, but gradually returns to basal levels within 10 minutes despite the continued presence of vasopressin (Hamada et al., 1992a; Nathanson et al., 1992a; Bygrave et al., 1994). These transient responses to stimulation with vasopressin are detected only if the bile volume is sampled at intervals of 1 minute or less.

In light of the apparent requirement of Ca^{2+} in bile flow, measurements of changes in perfusate Ca^{2+} concentration utilising a Ca^{2+}-sensitive electrode as well as changes in bile calcium concentration (measured by atomic absorption spectroscopy) have been made concomitant with these measurements of bile flow (see Fig.1 in Hamada et al., 1992a). Indications from these studies are that vasopressin increases biliary calcium concentration (Hamada et al., 1992a). In intact rats, the bile calcium concentration has been

shown to increase in response to an oral dose of Ca^{2+} (Yamaguchi and Sugii, 1980). Additionally, calcitonin, a hormone which is involved in the regulation of blood Ca^{2+} concentrations by antagonizing many of the effects of calcitriol and parathyroid hormone (see Part 1, Section (D) (I)), has been shown to increase liver and biliary Ca^{2+} concentration (Yamaguchi and Yamamoto, 1981; Yamaguchi and Imase, 1988)). The mechanism for this putative route of Ca^{2+} excretion may involve the calcitonin receptor-mediated Ca^{2+} influx (Yamaguchi and Ito, 1985), the transcellular passage of this Ca^{2+} via the Ca^{2+}-sequestering organelles, and the release of the Ca^{2+} into the bile. The perfusate Ca^{2+} changes follow the pattern indicated in Fig.2 in Hamada et al. (1992a), as shown by Altin and Bygrave (1985), and indicate that a large amount of Ca^{2+} is taken up by the liver under these conditions. While the endoplasmic reticulum and the mitochondria are able to sequester a large amount of Ca^{2+} under these conditions (see e.g. Altin and Bygrave, 1986), the possibility that some Ca^{2+} is released into bile cannot be excluded.

Some evidence for a role for the liver in the regulation of blood Ca^{2+} concentration includes the observation that ligation of the bile duct appears to increase blood Ca^{2+} concentrations (Yamaguchi, 1978; Yamaguchi and Yamamoto, 1978), and that administration of an oral dose of $^{45}Ca^{2+}$, which increased the blood Ca^{2+} concentration, resulted in a larger $^{45}Ca^{2+}$ concentration in the faeces than in the other Ca^{2+} stores (Yamaguchi et al., 1979). Biliary calcium has been shown to be of canalicular origin and related to bile acid secretion (Cummings and Hoffmann, 1984). It has been estimated that 80% of the Ca^{2+} in the bile is derived via the transcellular route (Limlomwongse et al., 1988). These data are also in harmony with another view, whereby under basal conditions Ca^{2+} enters the bile paracellularly, and part of this is taken up into the hepatocytes via canalicular Ca^{2+} channels, and subsequently excreted at the sinusoidal pole (Hill et al., 1985). Perturbation of this Ca^{2+} flux, either by the action of a Ca^{2+}-mobilizing agonist such as adrenaline or a Ca^{2+}-channel blocker such as verapamil, results in an increased canalicular Ca^{2+} concentration (Hill et al., 1985). However, another possibility is that these agents affect secretory events, whereby the Ca^{2+} detected is that released in conjunction with other components, such as the bile acids.

(c) The effect of glucagon

Glucagon has been shown to be choleretic by various techniques, including the perfused rat liver (Graf, 1976; Thomsen and Larsen, 1981, 1983; Yamatani et al., 1985; Lowe et al., 1988), and *in vivo* in various species including the rat (Thomsen and Larsen, 1981, 1982; Romanski and Bochenek, 1983), guinea pig (Lenzen et al., 1990) and in humans (Dyck and Janowitz, 1971; Branum et al., 1990, 1991). Thus glucagon has been suggested to have a major role in the regulation of bile flow in humans and other species (Branum et al., 1991). This choleretic effect has been mimicked by the addition of various membrane permeant analogues of cyclic AMP, as well as by infusing forskolin, a potent activator of adenylate cyclase (Hamlin et al., 1990; Lenzen et al., 1990). The choleresis is of canalicular rather than ductal origin (Lenzen et al., 1990), and requires an intact microtubular system (Lenzen et al., 1990; Hayakawa et al., 1990; Hoshino et al., 1993). Glucagon has been shown to affect both bile acid-dependent and bile acid -independent bile flow. Concomitant with the former is an increase in bile acid secretion (Hayakawa et al., 1990; Hamlin et al., 1990), which may be the result of the stimulation of the activity of the Na^+ dependent bile acid transporter (Grüne et al., 1993) as well as the microtubular vesicular system (Hayakawa et al., 1990, 1993). In addition, stimulation of the Na^+,K^+-ATPase by glucagon has been suggested (Thomsen and Larsen, 1982).

Determinations of bile flow rate at intervals smaller than those utilised in the above studies have shown that within the first 5 minutes of glucagon administration, it evokes a significant diminution in flow that returns to levels slightly greater than basal despite the continued presence of the hormone (Hamada et al., 1992a; Bygrave et al., 1994). This is accompanied by a slight efflux of Ca^{2+} into the perfusate (Altin and Bygrave, 1986; Hamada et al., 1992a). No significant change in bile calcium has been detected. The nature of this initial cholestatic response is presently unclear, but the timing of this event coincides with the release of Ca^{2+} mentioned above. While inhibition of disassembly of the actin filaments has been shown with cyclic AMP (Cheek and Burgoyne, 1987; Hamlin et al., 1990), variable data on the effects of glucagon and cyclic AMP on the permeability of tight junctions have been presented.

(d) The role of signalling cross-talk

The synergistic action of glucagon and Ca^{2+}-mobilizing hormones is known to lead to a considerably enhanced influx of Ca^{2+} (see Part I, Section (D) (V) for a discussion). The effect of cross-talk between these signalling systems on bile flow has recently been examined (Hamada et al., 1992a). As indicated in Fig.2 in Hamada et al. (1992a), while the duration of the transient increase in bile flow appears to be similar to that in the presence of vasopressin alone, its magnitude is enhanced to approximately 2.5 times the basal rate (see also Bygrave et al., 1994). As with vasopressin alone, the bile flow rate subsequently decreases, but is sustained at this lower rate for the duration of the hormone infusion (Hamada et al., 1992a; Bygrave et al., 1994). Again, there are indications that the concentration of Ca^{2+} is increased at the transient peak (Hamada et al., 1992a). The effects of vasopressin on bile flow are, as found with Ca^{2+} mobilization, greatly modified by the prior addition of glucagon. This finding further supports the notion that intracellular Ca^{2+} mobilization has a role in regulating the flow of bile. Apart from the transient cholestatic action induced by glucagon alone, in the presence of this hormone there is an augmentation of the vasopressin-induced choleresis, as well as a delay in the recovery to basal rates of flow following the vasopressin-induced decrease in bile flow (Bygrave et al., 1994). These experiments provide evidence that cross-talk in liver between the second messenger-mediated signalling systems activated by glucagon and vasopressin (see Part I, Section (D) (V)), may be an important modulator of bile flow (Bygrave et al., 1994).

The mechanism by which this crosstalk serves to stimulate the flow of bile is not known. The effects could be a direct consequence of the changes in Ca^{2+} fluxes observed under these conditions (see Part 1, Section (D) (V)).

A number of intracellular events appear to be influenced by increased intracellular Ca^{2+} concentration. One of these is an increased rate of canalicular contractility. Actin is present in the hepatocyte as part of the microfilamentous network (for a review see e.g. Phillips et al., 1983). The microfilaments are highly concentrated in the canalicular region of the hepatocyte, and at least the portion of these in close apposition with the canalicular

membrane appear to interact with the tight junctions (Tsukada et al., 1994). Thus contraction of the microfilaments in the peri-canalicular region of the cells results in a decrease in the volume of the canaliculus (see Fig.3, Section (C) of this Review). Canalicular contractions have also been shown in cell culture (Kawahara et al., 1990) and their demonstration *in vivo* is evidence for a physiological role for this event (Watanabe et al., 1991), presumably to propel the bile toward the ductules (French, 1985). Contraction has been shown to be induced as a result of the myosin light-chain kinase (MLC kinase)-mediated (Yamaguchi et al., 1991) phosphorylation of the myosin light chain, and the subsequent interaction of myosin with F-actin (filamentous or polymerised actin) (Kitamura et al., 1991). MLC kinase is activated by Ca^{2+}/calmodulin-mediated phosphorylation (Tsukada et al., 1994), and the timing of these events tend to coincide with the onset of contractility (Yamaguchi et al., 1991). These events are impaired under conditions of oxidative stress (Lemasters et al., 1983; Wilton et al., 1993; Stone et al., 1994).

A number of putative transport systems for the translocation of bile acids into the cell are present on the sinusoidal membrane. Of these, the Na^+-dependent bile acid co-transporter is probably the most important physiologically. This co-transports Na^+ with a bile acid molecule (Inoue et al., 1982), and therefore its activity is indirectly coupled to that of the Na^+,K^+-ATPase (see e.g. Frimmer and Ziegler, 1988; Zimmerli et al., 1989; Kast et al., 1994). This transport system is stimulated by glucagon and cyclic AMP (Edmondson et al., 1985; Botham and Suckling, 1986), an effect which has been attributed to cyclic AMP-mediated hyperpolarization of the hepatocyte membrane (Edmondson et al., 1985). This stimulation is mediated by protein kinase A, and is further potentiated by Ca^{2+}/calmodulin-dependent processes (Grüne et al., 1993) but decreased by protein kinase C action (Grüne et al., 1993, Divald et al., 1994).

The movement of vesicles from the sinusoidal to the canalicular pole of the hepatocytes is thought to be involved in transporting various components, including bile acids, from the sinusoidal to the canalicular pole. The microtubule-dependent vesicular transport system has been shown to be under hormonal control; vesicular transport is stimulated by cyclic AMP and

potentiated by Ca^{2+} (Hayakawa et al., 1990, 1992; Hoshino et al., 1993), but the effect of protein kinase C is less clear (Bruck et al., 1994). An important factor involves the trafficking of these vesicles, and it has been suggested that protein kinase A-mediated phosphorylation stimulates the targeting of vesicles to the canalicular domain (Hayakawa, 1990). It has been proposed that co-transport of bile acids and biliary lipids for excretion occurs by this pathway (Crawford et al., 1988). Both protein kinase C (Bruck et al., 1994) and Ca^{2+} (Beuers et al., 1993a) have also been implicated in the stimulation of vesicular exocytosis in hepatocytes.

Movement of vesicles across the hepatocyte is an ATP-dependent process, which involves the activity of molecular motors such as kinesin and dynein, the presence of both of which in hepatocytes has been shown (Marks et al., 1995; Oda et al., 1995). Kinesin has been shown to be modulated by phosphorylation with protein kinase A as well as by calmodulin (Crawford et al., 1994), possibly explaining the stimulation of vesicular movements by both cyclic AMP and increased intracellular Ca^{2+} concentration. Therefore the transport of a basal flux of these vesicles could be stimulated by glucagon, as well as by Ca^{2+}.

Vasopressin and angiotensin II have both been shown to induce efflux of glutathione (Raiford and Michell, 1991), an organic anion with a very high osmotic activity, and therefore an important component of bile-acid independent bile flow. Furthermore, small increases in cyclic AMP induced an increase in the rate of efflux of glutathione, while large increases have been shown to be inhibitory (Fernandez-Checa et al., 1993).

Since both glucagon and vasopressin are able to influence components involved in the flow of bile individually, some additive effects of these may be involved in addition to the synergism observed.

Finally, the synthesis of bile acids is now evidence to suggest that the activity of cholesterol-7-α-hydroxylase is subject to short-term regulation by hormones. The enzyme has been shown to be activated by cyclic AMP (Vlahcevic et al., 1991) and a general increase in the rate of synthesis of bile acids occurs in the presence of cyclic AMP and glucagon (Tang and Chiang, 1986; Vlahcevic et al., 1991; Chiang and Li, 1994).

Thus cross-talk-induced increases in bile flow potentially involves numerous factors (see Fig.6). The importance of each in these events forms part of the present investigation.

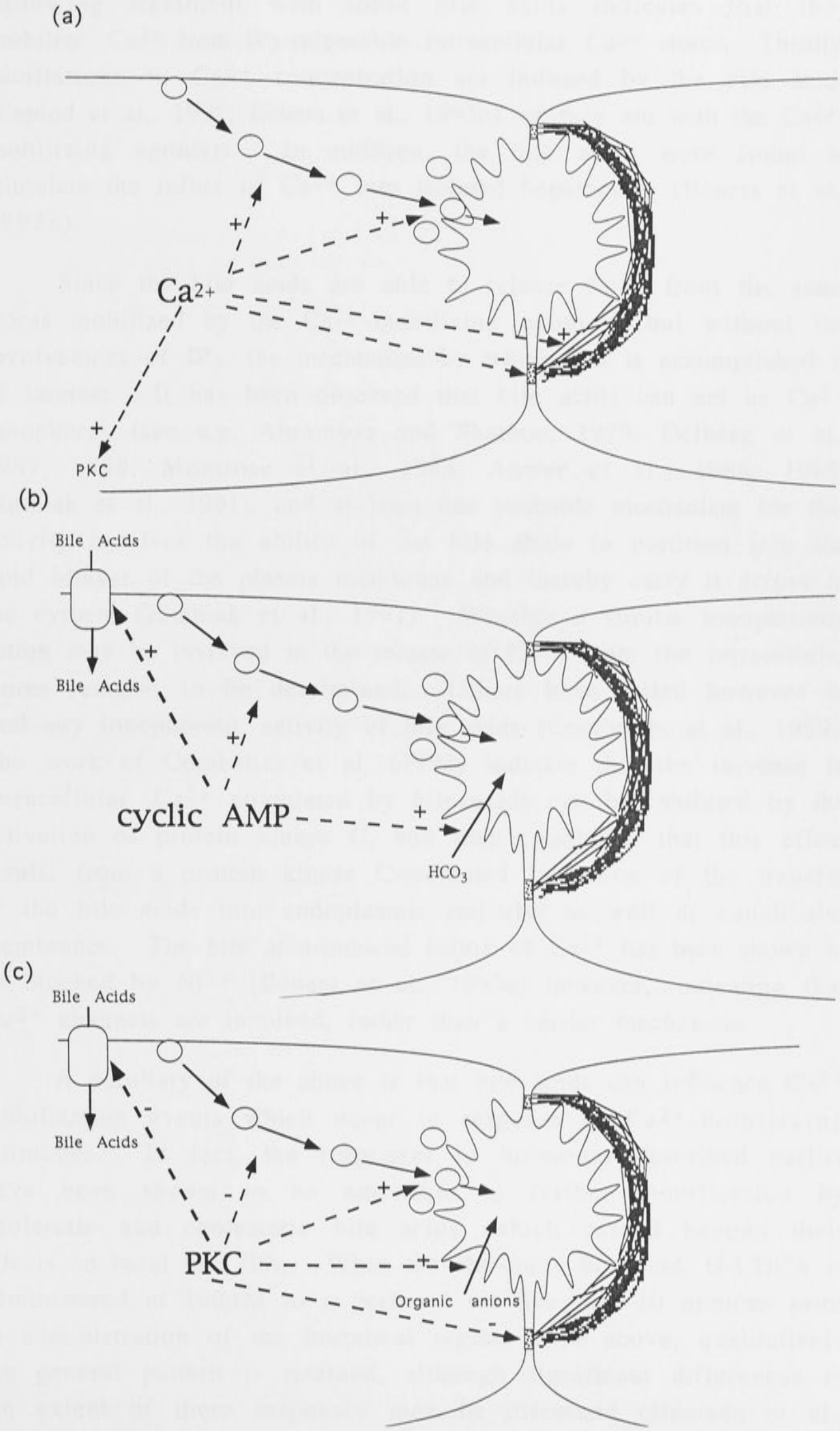
(e) Influence of bile acids on Ca^{2+} fluxes

Various studies have shown that at least some bile acids are able to mobilize Ca^{2+} in a number of cell types (Oelberg et al., 1987; Beuers et al., 1990; Coquil et al, 1991). A considerably greater mobilization of Ca^{2+} has been observed in hepatocytes (Bouscarel et al., 1993), probably because their uptake into this cell type is facilitated by bile acid transporters on their plasma membrane, rather than to a specific sensitivity to Ca^{2+} of hepatocytes; this results in a greater intracellular bile acid concentration in this cell type. Conjugates of bile acids may differ in their effects on intracellular Ca^{2+} mobilization, and this may be explained partly by differences in their uptake into the cell. Some groups have found that both choleretic and cholestatic bile acids mobilize Ca^{2+} (Bouscarel et al., 1993). It has been suggested however, that the mechanism by which they do this differ (Nathanson, 1994). Only some bile acids appear to induce a sustained increase in intracellular Ca^{2+} concentration, which is an indication that influx of the ion has been stimulated. Others have only found Ca^{2+} mobilization with cholestatic bile acids alone (Thibault and Ballet, 1993; Hamada et al., 1992b), which in fact has been ameliorated by the choleretic bile acids (Thibault et al., 1993). The role of the Ca^{2+} mobilized by the bile acids however is currently controversial. Therefore while there is evidence to suggest that the Ca^{2+}-releasing activity could be relevant to their cholestatic potential (Combettes et al., 1989; Hamada et al., 1992b), this is not clear. On the other hand, it has been shown that Ca^{2+} mobilized by the bile acids is either not relevant to their action on bile flow (Farrell et al., 1990), or even serves to facilitate certain choleretic actions of bile acids, such as vesicular exocytosis (see e.g. Beuers et al., 1993a).

Investigations have been carried out into the source of the Ca^{2+} released in this way. Firstly, the choleretic bile acids UDCA, and T-UDCA do not induce the formation of IP_3 (Combettes et al., 1989; Beuers et al., 1993b; Bouscarel et al., 1993). Secondly, the observation that no further Ca^{2+} is mobilized by phenylephrine (Beuers et al., 1993b) or vasopressin (Bouscarel et al., 1993)

Fig.6. Main features of the direct role of the individual signalling pathways stimulated by vasopressin and glucagon on the flow of bile.

The anatomical detail is as described in the legend to Fig.3. The + and - refer to the stimulatory and inhibitory effects of these on the flow of bile. Effects of Ca^{2+} which result in the stimulation of the flow of bile are shown in Fig.6a. The movement of vesicles, as well as their release into the bile canaliculus is stimulated as a result of increases in intracellular Ca^{2+} concentration. Additionally, Ca^{2+} stimulates the contractility of the bile canaliculus. However, the increased paracellular permeability promoted by the ion decreases the bile flow rate. The events stimulated by cyclic AMP are shown in Fig.6b, the most notable of which are the stimulation of bile acid uptake, vesicular movements and the activity of certain ion transporters. As indicated in Fig.6c, stimulation of the activity of protein kinase C is thought to inhibit some of the events involved in facilitating the flow of bile, while at the same time stimulation of both vesicular exocytosis and canalicular organic anion transport has been shown. For further details see text.


(a)
Ca2+
PKC
(b)
Bile Acids
Bile Acids
cyclic AMP
HCO3
(c)
Bile Acids
Bile Acids
PKC
Organic anions

following treatment with these bile acids indicates that they mobilize Ca^{2+} from IP_3-releasable intracellular Ca^{2+} stores. Thirdly, oscillations in Ca^{2+} concentration are induced by the bile acids (Capiod et al., 1991; Beuers et al., 1993b), as they are with the Ca^{2+}-mobilizing agonists. In addition, the bile acids were found to stimulate the influx of Ca^{2+} into isolated hepatocytes (Beuers et al., 1993a).

Since the bile acids are able to release Ca^{2+} from the same stores mobilized by the Ca^{2+}-mobilizing agonists, but without the involvement of IP_3, the mechanism by which this is accomplished is of interest. It has been observed that bile acids can act as Ca^{2+} ionophores (see e.g. Abramson and Shamoo, 1979; Oelberg et al., 1987, 1988; Montrose et al., 1988; Anwer et al., 1988, 1989; Zimniak et al., 1991), and at least one probable mechanism for this activity involves the ability of the bile acids to partition into the lipid bilayer of the plasma membrane and thereby carry it across to the cytosol (Zimniak et al., 1991). Whether a similar ionophoretic action may be involved in the release of Ca^{2+} from the intracellular stores remains to be determined. Others have failed however to find any ionophoretic activity of bile acids (Combettes et al., 1989). The work of Combettes et al. (1992) indicate that the increase in intracellular Ca^{2+} stimulated by bile acids can be inhibited by the activation of protein kinase C, and they concluded that this effect results from a protein kinase C-mediated inhibition of the transfer of the bile acids into endoplasmic reticular as well as canalicular membranes. The bile acid-induced influx of Ca^{2+} has been shown to be blocked by Ni^{2+} (Beuers et al., 1993a) however, indicating that Ca^{2+} channels are involved, rather than a carrier mechanism.

A corollary of the above is that bile acids can influence Ca^{2+} mobilization events which occur in response to Ca^{2+}-mobilizing hormones. In fact, the responses to hormones described earlier have been shown to be amenable to further modification by choleretic and cholestatic bile acids, which extend beyond their effects on basal bile flow. When the choleretic bile acid, G-UDCA is administered at 100μM to a perfused rat liver for 10 minutes prior to administration of the hormonal regime used above, qualitatively the general pattern is retained, although significant differences in the extent of these responses may be discerned (Hamada et al., 1992b). As indicated in Fig.3 in Hamada et al. (1992b), the major

effects are an increase in the peak rate of bile flow, as well as a decreased duration of the subsequent diminution of bile flow. When glucagon is co-administered with vasopressin, the main feature is the marked increase in the peak bile flow rate. Similarly, the general pattern of perfusate Ca^{2+} changes is largely unchanged except for a tendency towards further Ca^{2+} inflow (Hamada et al., 1992b, Bygrave et al., 1994). Whether this increased influx is relevant to the mechanism by which they induce bile flow is unclear.

The hormone-induced responses in bile flow following prior administration of the cholestatic bile acid T-CDCA at the same 100μM concentration are shown in Fig.5 in Hamada et al. (1992b). It may be noted that in many respects they are opposite to those seen with G-UDCA. The most notable feature is the virtual absence of any peak induced by co-administration of glucagon plus vasopressin. Additionally, at the beginning of the T-CDCA infusion there is a noticeable release of Ca^{2+} from the liver into the perfusate, and since this is accompanied by metabolic changes such as an increase in glucose output and oxygen consumption, this reflects a release of the ion from intracellular storage sites (see Fig.5, Hamada et al., 1992b; Bygrave et al., 1994). As discussed earlier, this feature is characteristic of a number of bile acids, and in the absence of any Ca^{2+} release from the perfused liver being induced by the choleretic bile acid, it is tempting to speculate that this is related to the mechanism by which the cholestatic bile acids induce cholestasis (Combettes et al., 1992; Thibault et al., 1993; Hamada et al., 1994). The opposite nature of the responses in bile flow also extend to perfusate Ca^{2+} fluxes. In the presence of T-CDCA, the Ca^{2+} influx induced by vasopressin in the presence or absence of glucagon is significantly attenuated. In fact, there is a tendency towards the release of Ca^{2+}, a feature reflected not only in the decreased amount of Ca^{2+} taken up, but also in the increased amount released (see Fig.4 in Hamada et al., 1992b).

These data point to an effect on intracellular Ca^{2+} homeostasis, which may be specific to stimulation by cholestatic and choleretic bile acids. Whether the facilitation of the influx due to G-UDCA is related to an ability to clear intracellular Ca^{2+}, thus decreasing feedback action by the ion on influx mechanisms, and whether the

opposite is the case with the cholestatic bile acid, is presently unclear.

(III) Cholestasis

The term cholestasis describes a clinical syndrome in which bile secretion is decreased. Like the flow of bile, its decrease in cholestasis appears to be complex, involving numerous factors. Cholestasis can be induced by a number of chemical interventions, and this has been utilised in the development of a number of animal models for cholestasis. Common observations in many of these models include decreased membrane fluidity, decreased plasma membrane transporter activity, decreased canalicular contractility, and an increased paracellular permeability. Many of these aberrations can vary considerably between the models used. For example changed immunostaining of the tight junction protein ZO-1 has been observed in the bile duct ligation model, but not in cholestasis induced by ethynylestradiol (Anderson et al., 1989; Reichen, 1993a,b), perhaps reflecting the difference in severity of the effect on the tight junctions in cholestasis induced in these models (Landmann, 1995).

The bile duct ligation model is simple, and serves as a model for extrahepatic cholestasis. However some principles are applicable generally to cholestatic conditions. The stasis of bile in the canaliculus can itself influence events at the canalicular membrane, and some of these effects may be translated to the sinusoidal membrane, especially since this form of cholestasis may result in the loss of polarity of the cell (Fricker et al., 1989), possibly as a result of a severe loss of tight junctional integrity (Accatino et al., 1981).

Intrahepatic cholestasis can be induced by various chemicals (see e.g. table in Reichen and Simon, 1988). The most commonly used experimental models for intrahepatic cholestasis are those employing cholestatic bile acids, such as taurochenodeoxycholate (T-CDCA), some estrogen derivatives such as ethynylestradiol and estradiol-17ß-glucuronide, and agents which disrupt cytoskeletal activity, such as phalloidin and cytochalasin B. Additionally, hormonal stimulation can induce transient cholestatic events (Hamada et al., 1992a,b; Nathanson et al., 1992).

Cholestasis can be induced by agents which specifically act on the microfilamentous system (Phillips et al., 1975; Dubin et al., 1978), such as phalloidin or cytochalasin B; thus the importance of this system in the flow of bile is further suggested. Phalloidin is a cyclic heptapeptide derived from the mushroom *Amanita Phalloides* (for a review see e.g. Frimmer, 1987). The cholestatic potential of phalloidin was first shown by Matschinsky et al. (1960), who showed in the perfused liver system, that complete cessation of bile flow can be rapidly induced when high concentrations of phalloidin (e.g. 20 μM) are infused. Phalloidin induces cholestasis in vitro (Rahman and Coleman, 1986) and is known to stimulate the irreversible polymerization of F-actin (Wieland et al., 1977). Therefore, assuming minimal non-specific effects of phalloidin, the cholestasis produced by this agent is directly related to disruption of the microfilaments. In fact, treatment with phalloidin does result in decreased canalicular contractility (Watanabe et al., 1983), increased tight junctional permeability (Elias et al., 1980) and impairment of secretory events (Rahman and Coleman, 1986). Further, it has been found that this agent induces blebbing of the surface of the canaliculus, indicating plasma membrane collapse (Boyer, 1987). Some evidence exists however, for phalloidin-induced inhibition of the interaction between myosin and actin (Tuchweber et al., 1981).

Derivatives of estradiol have commonly been utilised to serve as models for cholestatic conditions. Since cholestasis often occurs in pregnancy (Svanborg, 1954; Vore, 1987), the model has direct clinical relevance. The mechanism by which cholestasis is induced by estradiol is however presently unclear. Cholestasis may be induced by injecting the animal with ethynylestradiol *in vivo*, usually for 5 days, or estradiol-17-ß-D-glucuronide either *in vivo* or *in vitro*. Other derivatives may also be utilised. It has been established that the increase in paracellular permeability associated with this treatment (Kan et al., 1989) actually follows the decrease in bile flow (Jaeschke et al., 1987).

A number of studies has also indicated that there is a decrease in bile acid-independent bile flow. Evidence for this is the fact that the concentration of taurocholate has been shown to increase following cholestasis (Gumucio and Valdevieso, 1971; Jaeschke et al., 1987). Indications are that treatment with

ethynylestradiol inhibits ATP-dependent transport systems, but not electrogenic transport systems (Arias et al., 1994). The activity of the Na^+,K^+-ATPase is decreased in cholestasis induced by ethynylestradiol (Layden and Boyer, 1976; Reichen and Paumgartner, 1977), but also in cholestasis induced by chlorpromazine (Keeffe et al., 1980), and the bile acid taurolithocholate (Reichen and Paumgartner, 1979) as well as bile duct ligation (Schmelck etal., 1979). This effect is thought to be secondary to decreased fluidity of the sinusoidal membrane (Davis et al., 1978; Berr et al., 1984), resulting from alterations in its lipid and protein composition in these cholestatic conditions. For example, cholesterol is found to be incorporated into membranes to a greater extent in cholestasis (see e.g. Schachter, 1984). Thus even though bile flow largely involves canalicular events, it can be significantly affected by a lesion at the sinusoidal membrane (Hornstein et al., 1992). Whether part of the cholestasis results from a direct effect on the bile acid and organic anion transporters, or is secondary to effects on other transporters, such as the Na^+,K^+-ATPase (see e.g. Bossard et al., 1993), needs to be clarified.

A difficulty associated with the analysis of cholestatic phenomena is in differentiating between the cause and effect. Therefore while many of the features mentioned above occur in cholestasis, it is often less clear whether this has induced the cholestasis or whether the cholestasis which has developed from a separate cause has induced this effect. With improvements in the resolution in the techniques utilised in these studies however, temporal relationships between these events may be dissected.

The tight junctions

Components of bile and plasma can exchange either as a result of paracellular or transcellular transport between these compartments. The permeability of the paracellular pathway is determined mainly by the status of the tight junctions (see Fig.3, Section (C)), and since the integrity of this barrier is decreased in cholestasis, the nature of this barrier will be discussed briefly. In studies of changes in permeability of the paracellular pathway the most important technique utilised has been that where the relative rate of appearance in bile of a bolus of compounds which are readily detectable in bile. For example horse radish peroxidase

(HRP) has been shown to appear in bile in two phases, the first phase representing transport across the paracellular pathway, and the later peak representing transcytosis. Hormones such as vasopressin have been shown to induce an increase in the first phase, and this has been interpreted as a vasopressin-mediated increase in tight junctional permeability (Lowe et al., 1985, 1988). Other studies have also indicated increased paracellular permeability with agents which mobilize Ca^{2+}, such as BHQ (Llopis et al., 1990; Nathanson et al., 1992a) and A23187 (Kan and Coleman, 1988; Nathanson et al., 1992a), as well as stimulators of protein kinase C (Bruck et al., 1994). Thus the tight junctions are thought to be dynamic structures, their permeability being amenable to modulation by hormones (Lowe et al., 1988).

However, some caution may be required in interpreting these data, as it has been shown that transcellular vesicular transport of some compounds can be rapid (Graf, 1990). Additionally, the tight junctions are effective in inhibiting the back-diffusion of components of bile, and since the integrity of this barrier is an essential component of the osmotic theory of bile flow, the validity of the above observations has been questioned (Graf, 1990). Thus increased paracellular permeability is generally equated with decreased bile flow due to regurgitation of osmotically-active anions to the blood (Layden et al., 1978). However, the permeability of the tight junctions may apparently be increased in the absence of a decrease in bile flow (Kan and Coleman, 1986; Kan et al., 1989), and in some instances the increased paracellular permeability clearly follows the onset of cholestasis (Jaescke et al., 1987; Kan et al., 1989). Also, in some instances increased permeability of the tight junctions has been observed in conjunction with an increased bile flow rate in taurocholate-induced choleresis (Reichen, 1993a) and moderate hypocalcaemia (Graf, 1975; Reichen et al., 1985). The possibility of the presence of pores at the tight junctions cannot be totally ruled out.

(IV) Therapy of cholestatic conditions

It has been recognized both experimentally and clinically that the symptoms of cholestatic conditions such as primary biliary cirrhosis are ameliorated by treatment with bile acids. Particular bile acids are able to increase the bile flow rate as well as some of

the biochemical characteristics associated with the condition. The most commonly used bile acids in these studies are ursodeoxycholic acid (UDCA) and its tauro- and glyco-amidated derivatives.

In the untreated animal, certain bile acids have a choleretic potential which is much greater than that expected from the rate of its secretion into the bile, a situation referred to as hypercholeresis (Dumont et al., 1980). The favoured explanation for this phenomenon is that the UDCA is taken up by the ductular cells, and transported back to the hepatocyte for release into the canaliculus (the cholehepatic shunt hypothesis see e.g. Gurantz and Hofmann, 1984; Gurantz et al., 1991). An alternative explanation relates to the stimulation of bicarbonate secretion by these bile acids (Moseley et al., 1987; Anwer, 1992). A consequence of this hypercholeretic potential is that the dose of the bile acid which needs to be administered for a therapeutic effect is lower; generally the toxicity associated with their administration is minimal. In fact, it has been suggested that treatment with these agents ameliorates some of the other symptoms associated with the cholestasis (see e.g. Jacquemin et al., 1993; Reichen, 1993b; Vandemeeberg et al., 1993; Leuschner, 1994).

A significant portion of the cellular toxicity associated with cholestasis may be secondary to the accumulation of hydrophobic bile acids within the hepatocytes (Greim et al., 1972; Heuman, 1993; Queneau and Montet, 1994), and resulting detergent action on the cellular membranes (Schölmerich et al., 1984; Queneau and Montet, 1994). Thus at least part of the therapeutic influence of these agents is probably related to changing the relative proportions of the bile acid load to one where less-toxic bile acids (such as the UDCA derivatives) predominate (Ohiwa et al., 1993).

The significance of the interaction of the bile acids with the action of the Ca^{2+}-mobilizing hormones, especially in the action of these on both the flow of bile and Ca^{2+} fluxes forms part of this work.

Section C

AIMS OF THIS STUDY

Aims of this study

The data described above indicate that bile flow is subject to short-term modulation by glucagon and the Ca^{2+}-mobilizing hormones, features of which are only apparent at high resolution. This thesis describes further investigations of the relationship between Ca^{2+} fluxes and bile flow events, incorporating two major strategies. First, in view of the differences in the pattern of Ca^{2+} fluxes induced by the Ca^{2+}-mobilizing agents in the presence and absence of glucagon (Altin and Bygrave, 1985, 1986), it was important to characterize the bile flow events induced by these agents. Employing the maximum resolution attainable, it was possible to correlate Ca^{2+} fluxes (and to some extent changes in oxygen consumption and glucose release) with phases of bile flow (Bygrave et al., 1994). Indications were that there is an order of potency involved in the stimulation of the transient increase in bile flow, whereby glucagon < vasopressin < glucagon + vasopressin. An examination of the effects of other hormone combinations allowed correlation between Ca^{2+} flux and bile flow events. Further, the importance of timing of changes in intracellular Ca^{2+} concentration reflected in bile flow events became apparent at higher resolution.

Flowing from this was the hypothesis that the role of mobilization of intracellular Ca^{2+} in these events could be differentiated from the effect of influx of Ca^{2+} from the extracellular medium. This was examined by utilizing two different strategies: (1) characterizing the inhibition by Ni^{2+} of the influx of Ca^{2+} induced by vasopressin in the presence of glucagon with the perfused liver; and (2) utilizing low concentrations of both glucagon and Ca^{2+}-mobilizing agonists. This enabled further insights into the role of cross-talk in these events.

The second major strategy arose from indications that choleretic and cholestatic bile acids can have different effects on Ca^{2+} fluxes (Hamada et al., 1992b), suggesting the hypothesis that aberrations in Ca^{2+} signalling may contribute to the onset of cholestasis. It was thus of interest to ascertain whether Ca^{2+} signalling and bile flow responses are affected in rats made cholestatic by other techniques, namely *in vivo* by ethynylestradiol and *in vitro* by phalloidin. The role of biliary Ca^{2+} fluxes was also examined in this context. Since certain bile acids have been shown to ameliorate clinical symptoms of cholestasis, and have been

experimentally shown to increase the bile flow rate, this work was followed by a study on the effect of different choleretic bile acids on experimentally-induced cholestasis. In view of the hypothesis stated above, particular attention was paid to the correlation between perfusate and biliary Ca^{2+} fluxes and bile flow events.

As will become evident, the strategies described above have yielded new information on the role of Ca^{2+} and Ca^{2+}-mobilizing agonists in the regulation of bile flow.

Section D

GENERAL METHODS

General Methods

Male Wistar-strain albino rats, initially weighing from 220 to 380g and having free access to food were used in all experiments. Rats were anaesthesised with sodium pentobarbitone (50mg/kg body wt). Livers were perfused with Krebs-Henseleit (Krebs and Henseleit, 1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1) and containing 1.3mM $CaCl_2$; the final oxygen concentration was approx. 1mM. Perfusions were conducted in a non-recirculating mode and the perfusate delivered at a constant volume of 35ml/min by means of a Gilson Minipuls 3 peristaltic pump. For each experiment the liver was first pre-perfused for at least 30min before the infusion of any hormone each of which was administered by a pump-driven infusion syringe. Details are contained in the legends to the figures. Additionally, a fine plastic cannula was inserted into the bile duct to enable collection of samples following administration of agents to the inflow cannula in the portal vein.

Perfusate Ca^{2+} measurements

The perfusate Ca^{2+} concentration was monitored continuously with a Radiometer F2112 Ca^{2+}-selective electrode in a flow-through chamber placed on the outflow side of the liver; this is described in detail elsewhere (Reinhart et al., 1982; Altin and Bygrave, 1985). The electrode was coupled to a Radiometer K801 reference electrode via an agarose/KCl salt-bridge, and the combined signals were fed via an Orion Ionalyzer to a recording device. In experiments where greater resolution was desired, the recording procedure was refined such that the data from the Ca^{2+}-selective electrode was fed, via a maclab recorder, into a computer. The data was then analysed using appropriate computer programs (such as Igor (Wavemetrics) or Excel (Microsoft)). In this way, changes in perfusate Ca^{2+} concentration in order of a few micromolar occurring in less than 1 second could be accurately monitored. Additionally, multiple experiments could be collated enabling an average Ca^{2+} trace to be presented.

Other measurements

Bile flow was measured by weighing the bile fluid collected in Eppendorf tubes every 5s, 20s or 60s as indicated in the legends to the figures; it was assumed that 1µl is equivalent to 1mg wet weight. Calculations carried out to determine the relative lag time in each of the perfusate and bile flow cannulae, showed that while the sum total of canalicular and ductular influences on bile flow were immediately translated to the bile flow cannula, the Ca^{2+} events lagged on average by 10s. Lag times were measured by injecting Ca^{2+} into the perfusate and measuring the time elapsed before a response was observed at the Ca^{2+} electrode. Dithionite was injected to similarly ascertain lag times to the oxygen electrode. These lag times were subtracted from the data prior to their presentation in the results section.

Chemicals and materials

Hormones were obtained from Sigma Chemical Co., St Louis, MO, USA, except glucagon, which was obtained from Eli Lilly Pty Ltd., Sydney, Australia for some series of experiments. Ca^{2+}-selective electrode membranes (F2112) and filling solutions (S43316) were obtained from Radiometer, Copenhagen, Denmark. Other chemicals used were of analytical reagent grade.

Expression of data

Ca^{2+} concentration changes are expressed as µmol/min per g of liver. An average value of 0.1µmol/min per g of liver corresponds to a change of perfusate Ca^{2+} of 40µM. Data from at least 3 independent experiments were used to compile the Ca^{2+} flux changes. All experiments were performed at least five times. Where indicated, data are expressed as means $\pm$ S.E.M. for the numbers of independent experiments described. Where necessary data were analysed for significance utilising the 2-tailed paired or unpaired t-test.

Section E

EXPERIMENTAL

Chapter 1

The synergistic action (cross-talk) of glucagon and vasopressin induces early bile flow and plasma-membrane calcium fluxes in the perfused rat liver

The synergistic action (cross-talk) of glucagon and vasopressin induces early bile flow and plasma-membrane calcium fluxes in the perfused rat liver

INTRODUCTION

An increase in the cytoplasmic free calcium (Ca^{2+}) concentration is recognised as being a key triggering event in secretory systems (for a recent review see Burgoyne and Morgan, 1993). Depending on the cell type, this increase in intracellular Ca^{2+} can be induced by a range of stimuli.

Evidence of a role for Ca^{2+} in modulation of bile flow is provided by the findings that the removal of Ca^{2+} from the extracellular medium results in its attenuation (Graf, 1975; Reichen et al., 1985; Stammler et al., 1990) and that hormones known to mobilise Ca^{2+} also are able to rapidly modulate such flow (Graf, 1975; Hill et al., 1985; Nathanson et al., 1992a; Hardison et al., 1991, Hamada et al., 1992a,b; reviewed in Nathanson & Boyer, 1991; Bygrave et al., 1994 and the Introductory review). The mechanism(s) by which bile flow is modulated by such hormonal action remains unclear although a number of possibilities have been suggested (see e.g. Nathanson & Boyer, 1991; Bygrave et al., 1994; Introductory Review).

The ability to measure small changes in perfusate Ca^{2+} in the perfused rat liver (Reinhart et al., 1982; Altin & Bygrave, 1985) concomitant with bile flow (Hamada et al., 1992a,b) has led to an investigation in further detail of the role of Ca^{2+} mobilisation in this event. Advantage was taken of knowledge that cross-talk between glucagon and vasopressin is able to rapidly modulate bile flow in the perfused rat liver (reviewed by Bygrave et al., 1994) consonant with its ability to modulate Ca^{2+} fluxes in the liver (reviewed by Bygrave & Benedetti, 1993). The sensitivity of this technique has been refined so as to enable an assessment to be made of the extent to which very rapid changes in rates of both bile flow and patterns of Ca^{2+} mobilisation occur simultaneously in the perfused rat liver (see the General Methods section). The data obtained provide

clearer insights into the relationship between plasma membrane Ca^{2+} fluxes and the modulation of bile flow in the intact organ.

EXPERIMENTAL

The experimental details are largely as indicated in the General Methods section. The calcium data were recorded by a Maclab recorder, based on a sample speed of 20 samples per s. Data were then transferred to a Microsoft Excel spreadsheet where an average value for each data point was calculated. The final results were then graphed. Oxygen uptake was measured as described previously (Hamada et al., 1992a).

RESULTS

As stated in the introductory section, the nature of the role of Ca^{2+} in bile flow remains elusive. An investigative route into this question however is one that takes account of knowledge that hormones with Ca^{2+}-mobilising actions are able to rapidly modulate bile flow and that glucagon (which on its own has less potent actions on Ca^{2+} mobilisation than Ca^{2+}-mobilising agonists [see e.g. Charest et al., 1983; Mauger & Claret, 1986]), in turn synergistically modulates the action of these Ca^{2+}-mobilising hormones (reviewed in Bygrave & Benedetti, 1993) through cross-talk between the signals generated by IP_3- and cyclic AMP-generating agonists (Bygrave et al., 1994).

Effects of vasopressin administration on plasma membrane Ca^{2+} fluxes and bile flow.

Data in Fig.1 illustrate the nature of the data obtainable following the improvements made to the technique for measuring both bile flow and net changes in perfusate Ca^{2+} in the perfused rat liver. Fig.1a shows the changes in perfusate Ca^{2+} that occur following the administration of vasopressin at zero time in the trace. Little change in perfusate Ca^{2+} is observed until 15s when net efflux of the ion commences. This efflux continues at a constant rate until 35s when the rate of Ca^{2+} efflux is maximal and then begins to decline. By approx. 55s, net Ca^{2+} influx has commenced and by approx. 80s this has reached maximal rates.

Fig.1. Rapid responses of perfusate Ca^{2+} and bile flow to vasopressin in the perfused rat liver.

Livers were perfused with Krebs-Henseleit bicarbonate medium containing 1.3mM Ca^{2+}. After a pre-perfusion period of 30 min, vasopressin (10nM) was infused from time zero in the panels shown. Fig.1a shows the average of 3 independent Ca^{2+} traces which were analysed as indicated in the Experimental section. The letters A to E are the marker time points referred to in Fig.1b. The data have been corrected for a lag of 10 $\pm$ 1s which is the time taken, in our experimental system, for Ca^{2+} to flow from the point of entry into the liver and then away from the liver to the Ca^{2+} electrode. Fig.1b shows the changes in bile flow, following the administration of vasopressin, sampled at 5s intervals. The average $\pm$ SEM from 6 independent experiments is shown.

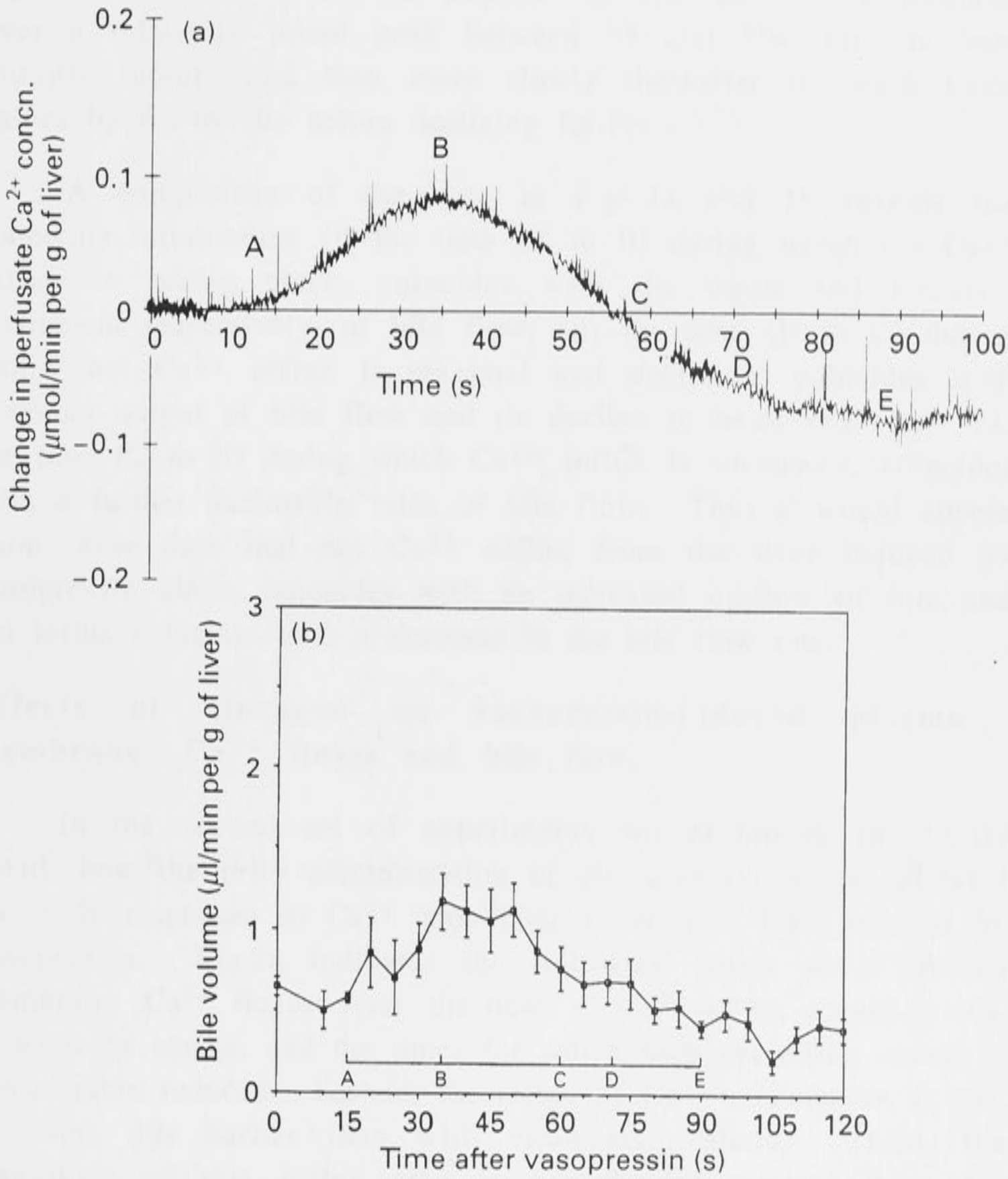

(a)
Change in perfusate Ca^{2+} concn. (μmol/min per g of liver)
0.2
0.1
0
−0.1
−0.2
A
B
C
D
E
0
10
20
30
40
50
60
70
80
90
100
Time (s)
(b)
Bile volume (μl/min per g of liver)
3
2
1
0
A
B
C
D
E
0
15
30
45
60
75
90
105
120
Time after vasopressin (s)

Fig.1b shows the concomitant changes in bile flow that occur every 5s following vasopressin administration to the perfused rat liver. In previous work we had sampled bile flow at 60s intervals (Hamada et al., 1992a,b). It is seen first, that the onset is not significant until 25 to 30s has elapsed. Second, bile flow is maximal over a relatively broad peak between 35 and 50s; this declines initially rapidly and then more slowly thereafter to reach basal values by 65 to 70s before declining further.

A comparison of the data in Figs.1a and 1b reveals the following information: (i) the time (A to B) during which net Ca^{2+} efflux is taking place, coincides with the onset and maximal attainment respectively, of bile flow; (ii) the time (B to C) during which net Ca^{2+} efflux is maximal and declining, coincides with maximal output of bile flow and its decline to basal rates and (iii) the time (C to E) during which Ca^{2+} influx is underway, coincides with a further decline in rates of bile flow. Thus it would appear from these data that net Ca^{2+} efflux from the liver induced by vasopressin alone, coincides with an increased outflow of bile and net influx coincides with a decrease in the bile flow rate.

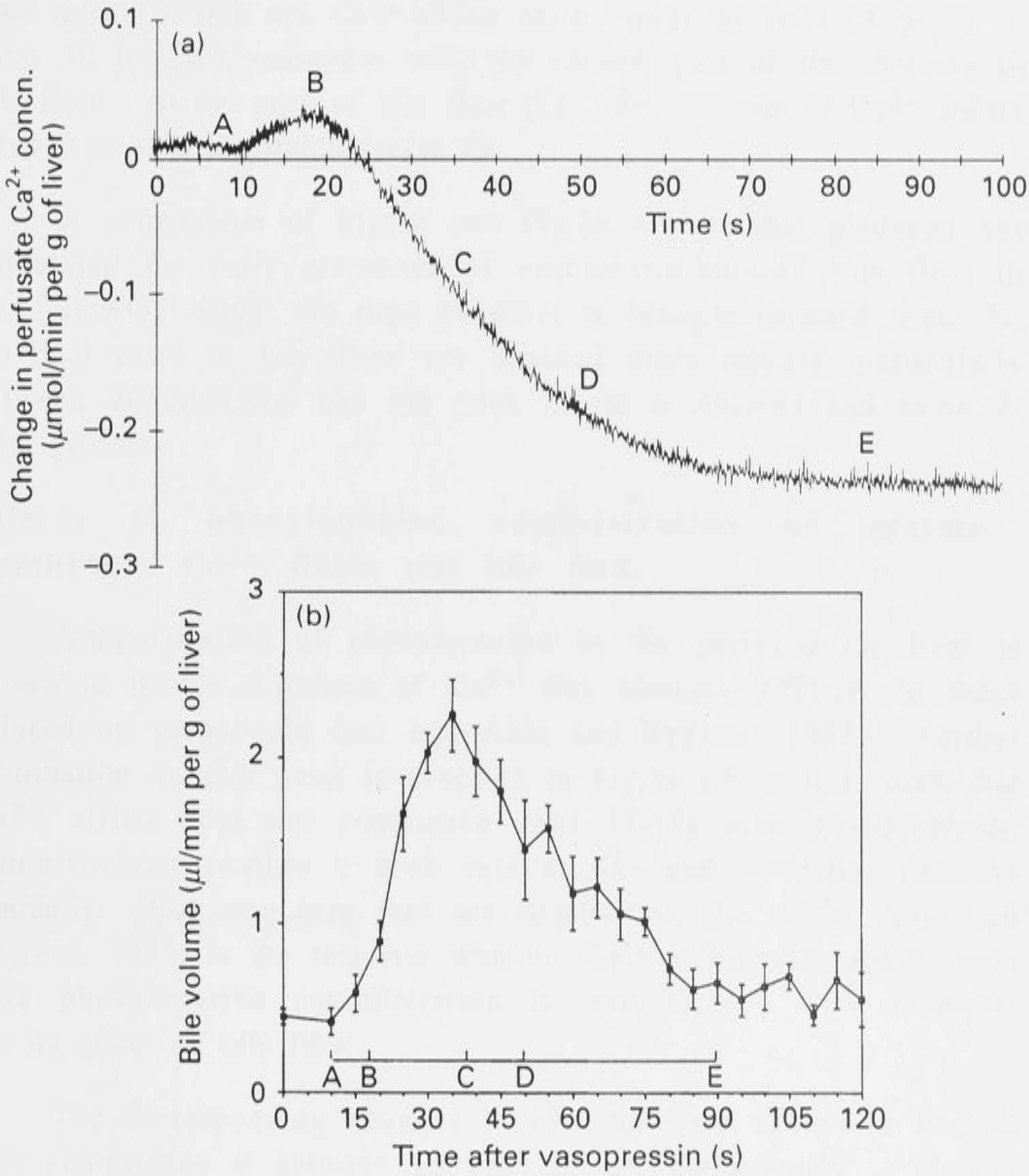
Effects of glucagon on vasopressin-induced plasma membrane Ca^{2+} fluxes and bile flow.

In the second set of experiments we examined, in similar detail, how the prior administration of glucagon for 4 min affected the early responses to Ca^{2+} mobilisation and bile flow induced by vasopressin. Fig.2a indicates the following points about plasma membrane Ca^{2+} fluxes: First, the onset of Ca^{2+} efflux occurs at 10s, or some 5s earlier, and the times for which such net efflux occurs is considerably reduced. Second, the influx of Ca^{2+} commences at 25s, or some 30s earlier than with vasopressin alone. Third, the magnitude of this influx also is considerably greater than that observed with vasopressin alone (see also Altin & Bygrave, 1986).

Data in Fig.2b show that the onset of vasopressin-induced bile flow is brought forward by prior administration of glucagon. It is underway by 15s and rapidly increases thereafter especially between 20 and 25s, peaks at 35s and declines to near basal values by 90s. There are indications that the decline exhibits a faster

Fig.2. Rapid responses of perfusate Ca^{2+} and bile flow to the synergistic action of glucagon and vasopressin in the perfused rat liver.

The experimental system was the same as that described in Fig.1 except that glucagon (10nM) was infused into the liver for 4 min prior to, and then concomitant with, the administration of vasopressin (10nM) which was commenced at time zero in the Figs. The Ca^{2+} trace data (average of 3 independent traces; Fig.2a) have been corrected for the lag referred to in the legend to Fig.1. The data for bile flow (Fig.2b) are the average $\pm$ SEM of 7 independent experiments. The letters A to E are the marker time points referred to in Fig.2b.


(a)
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Time (s)
Change in perfusate Ca^{2+} concn. (μmol/min per g of liver)
(b)
3
2
1
0
A
B
C
D
E
0
15
30
45
60
75
90
105
120
Time after vasopressin (s)
Bile volume (μl/min per g of liver)

phase between 35 and 50s and a slower phase from 50 to 90s (0.047 and 0.023µl/min^2 per g of liver respectively).

A comparison of the data in Fig.2a and Fig.2b reveals that the time during which net Ca^{2+} efflux occurs from the liver (A to B, ie. from 10 to 25s), coincides with the earliest part of the increase in bile flow. At the peak of bile flow (i.e. 35s), the rate of Ca^{2+} influx already is at a maximum (point C).

A comparison of Fig.1b and Fig.2b reveals that glucagon has modulated the early responses of vasopressin-induced bile flow in the following ways: the time of onset is brought forward some 7s, maximal rates of bile flow are attained more rapidly, particularly between 20 and 25s, and the peak height is sharper and some 2-fold greater.

Effects of phenylephrine administration on plasma membrane Ca^{2+} fluxes and bile flow.

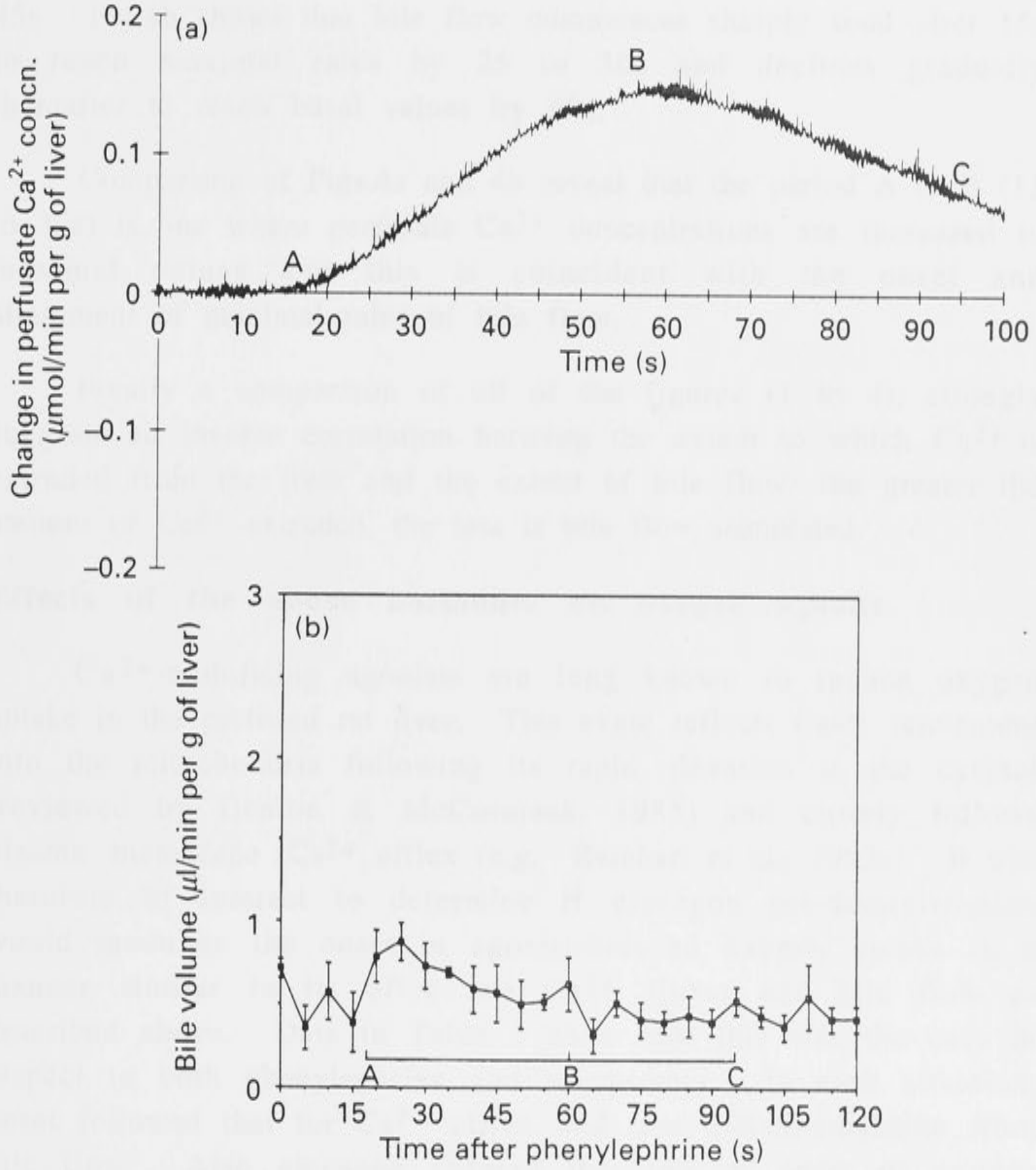
Administration of phenylephrine to the perfused rat liver is known to induce a pattern of Ca^{2+} flux changes different to those induced by vasopressin (see e.g. Altin and Bygrave, 1985). Further information on this point is revealed in Fig.3a where it is seen that Ca^{2+} efflux does not commence until 17-18s after phenylephrine administration, reaches a peak rate at 55s and thereafter declines gradually. Not seen here (but see Reinhart et al., 1982a; Altin and Bygrave, 1985) is the response wherein Ca^{2+} influx commences only after phenylephrine administration is terminated. This response has no effect on bile flow.

The corresponding changes in bile flow are shown in Fig.3b. This commences at between 15 and 20s and immediately is already maximal. However the degree of stimulation is relatively small and during the next 60s bile flow gradually diminishes to basal values. In some experiments, nitroprusside was pre-perfused to ensure that the changes induced by the agonist were not attributable to vasoconstrictive effects. Control experiments indicated that nitroprusside had little effect on basal bile flow.

A comparison of the data in Figs.3a and 3b reveal the point that although considerable Ca^{2+} has been extruded into the perfusate, bile flow has changed little.

Fig.3. Rapid responses of perfusate Ca^{2+} and bile flow to phenylephrine in the perfused rat liver.

The experimental system was the same as that described in Fig.1 except that after a pre-perfusion period of 30 min, phenylephrine (2μM) was infused from time zero in the panels shown. Fig.3a shows the average of 3 independent Ca^{2+} traces which were analysed as indicated in the Experimental section. The letters A to E are the marker time points referred to in Fig.3b. The data have been corrected for the lag referred to in the legend to Fig.1. Fig.3b shows the changes in bile flow, following the administration of phenylephrine, sampled at 5s intervals. The average ± SEM from 5 independent experiments is shown.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Change in perfusate Ca^{2+} concn. (μmol/min per g of liver)
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Time (s)
(b)
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Bile volume (μl/min per g of liver)
A
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60
75
90
105
120
Time after phenylephrine (s)

Effects of glucagon on phenylephrine-induced plasma membrane Ca^{2+} fluxes and bile flow.

Fig.4a reveals that glucagon reduces the onset of phenylephrine-induced Ca^{2+} efflux by 3-4s (cf. Fig.3a) as well as the magnitude of the efflux. Accordingly Ca^{2+} influx commences by 45s. Fig.4b shows that bile flow commences sharply soon after 15s to reach maximal rates by 25 to 30s and declines gradually thereafter to reach basal values by 80s.

Comparison of Figs.4a and 4b reveal that the period A to B (15 to 35s) is one where perfusate Ca^{2+} concentrations are increased to maximal values and this is coincident with the onset and attainment of maximal rates of bile flow.

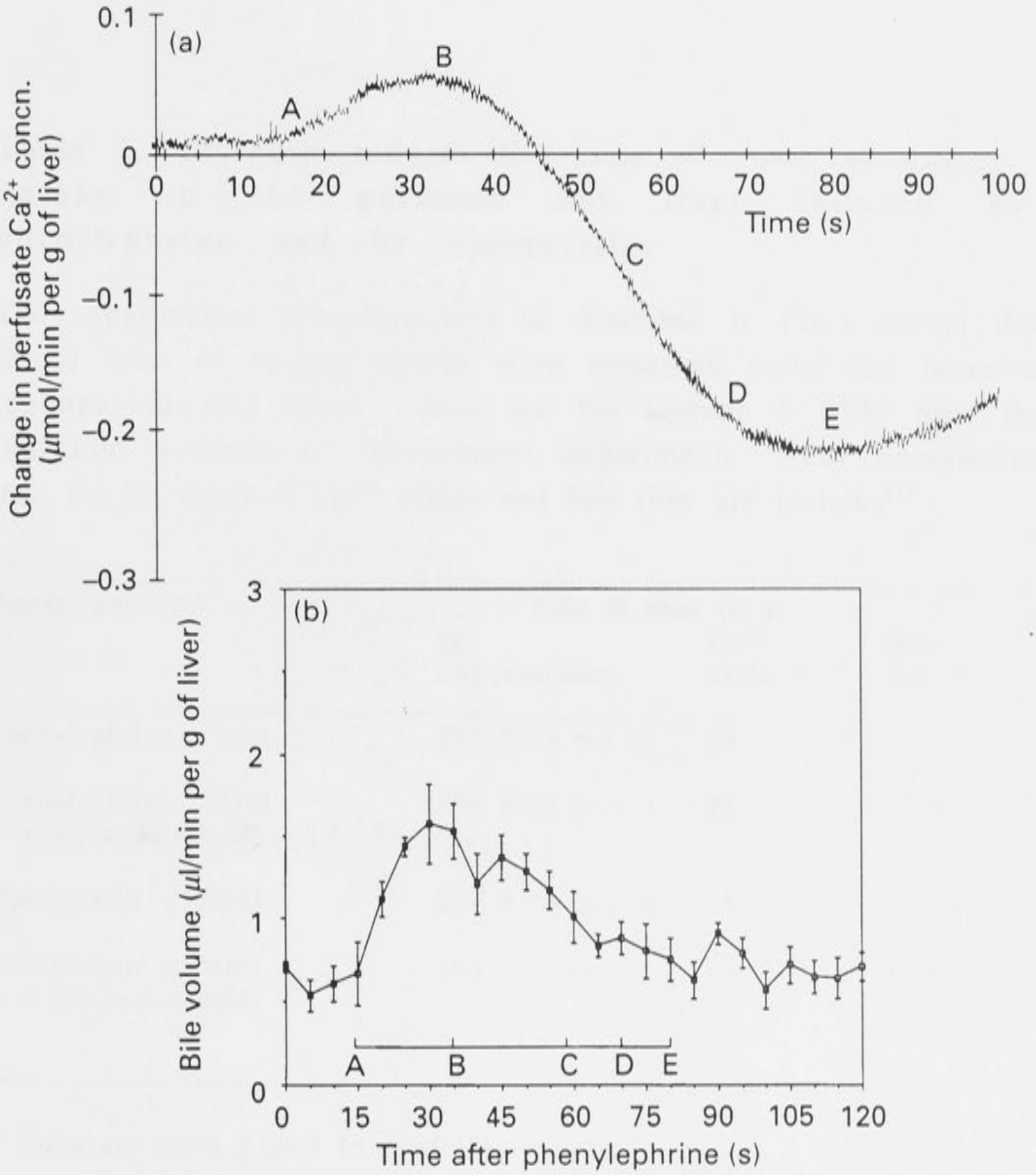
Finally a comparison of all of the figures (1 to 4), strongly suggests an inverse correlation between the extent to which Ca^{2+} is extruded from the liver and the extent of bile flow; the greater the amount of Ca^{2+} extruded, the less is bile flow stimulated.

Effects of the above hormones on oxygen uptake

Ca^{2+}-mobilising agonists are long known to induce oxygen uptake in the perfused rat liver. This event reflects Ca^{2+} movement into the mitochondria following its rapid elevation in the cytosol (reviewed by Denton & McCormack, 1985) and closely follows plasma membrane Ca^{2+} efflux (e.g. Reinhart et al., 1982a). It was therefore of interest to determine if glucagon pre-administration would modulate the onset in agonist-induced oxygen uptake in a manner similar to its effect on Ca^{2+} fluxes and bile flow as described above. Data in Table 1 show that this was the case in respect to both phenylephrine and vasopressin. In each situation, onset followed that for Ca^{2+} efflux and was indistinguishable from bile flow. Also glucagon reduced the time of onset of oxygen uptake induced by each agonist by 5 to 6s. The magnitude of oxygen uptake induced by phenylephrine or vasopressin however was similar in all four instances (data not shown).

Fig.4. Rapid responses of perfusate Ca^{2+} and bile flow to the synergistic action of glucagon and phenylephrine in the perfused rat liver.

The experimental system was the same as that described in Fig.1 except that after the 30 min preperfusion period glucagon (10nM) was infused into the liver for 4 min prior to, and then concomitant with, the administration of phenylephrine (2μM) which was commenced at time zero in the Figs. The Ca^{2+} trace data (average of 3 independent traces; Fig.4a) have been corrected for the lag referred to in the legend to Fig.1. The letters A to E are the marker time points referred to in Fig.4b. The data for bile flow (Fig.4b) are the average ± SEM of 7 independent experiments.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Time (s)
Change in perfusate Ca^{2+} concn. (μmol/min per g of liver)
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90
105
120
Time after phenylephrine (s)
Bile volume (μl/min per g of liver)

Table 1. Glucagon reduces the time of onset of oxygen uptake in the perfused rat liver induced by phenylephrine and by vasopressin.

The experimental procedure was as described in Fig.1 except that initial rates of oxygen uptake were measured under the hormone regimes indicated below. Data are the average ± SEM from the indicated numbers of independent experiments. For comparison data for the onset of Ca^{2+} efflux and bile flow are included.

Agent perfused	Time of onset (s) of		
	O_2 consumption	Ca^{2+} efflux*	Bile flow*
Phenylephrine (2μM)	24.5 ± 2.4 (n = 4)	18	15-20
Phenylephrine (2μM) + glucagon (10nM)	19.2 ± 0.6 (n = 4)	15	15-20
Vasopressin (10nM)	22.4 ± 2.6 (n = 3)	15	20-25
Vasopressin (10nM) + glucagon (10nM)	16.1 ± 1.5 (n = 4)	10	13-18

* Data are from Figs.1 to 4 of this chapter.

CONCLUSIONS

The experiments reported here provide fresh insights into a number of features concerning hormone-induced Ca^{2+} mobilisation and bile flow in rat liver. These insights arise first out of improvements to the Ca^{2+}-selective electrode technique (Reinhart et al., 1982a, Altin & Bygrave, 1985) that enable precise changes of the ion to be measured in the perfusate of a perfused rat liver system when physiological concentrations of extracellular Ca^{2+} are employed. The improvement made in this work was largely in the mode of detection and analysis of the Ca^{2+} signals generated. The second technical advance was to collect samples of bile at 5s intervals. This allowed information to be gained that is clearly unattainable with 60s (e.g. Hamada et al., 1992a,b; Hardison et al., 1991) or 30s (Nathanson et al., 1992) intervals of sampling.

Further insights into hormone-induced Ca^{2+}-mobilisation in liver

Data in this work provide further information about hormone-induced Ca^{2+} mobilisation in the intact perfused rat liver. First they reveal the precise times at which the onset of vasopressin-induced Ca^{2+} efflux occurs (15 $\pm$ 1s), the precise duration and magnitude of net Ca^{2+} efflux (35s; 35nmol of Ca^{2+} per g of liver, respectively), and the time of onset of net Ca^{2+} influx (55s) (see Fig.1a). The timing of these events may be compared with knowledge that vasopressin-induced increases in cytosolic Ca^{2+} measured in hepatocytes with Ca^{2+}-sensitive intracellular indicators commence at approx. 3s and reach maximal values by approx. 10s (see e.g. Charest et al., 1983; Combettes et al., 1986) and with knowledge that the onset of Ca^{2+} efflux in these cells measured with aequorin and 30mM extracellular Ca^{2+}, occurs within several seconds of vasopressin administration (Duddy et al., 1989). Direct measurement of changes in intracellular Ca^{2+} in the perfused rat liver is not easy (see Ruttner et al., 1993) but the changes in oxygen output induced by vasopressin reflecting Ca^{2+} uptake into the mitochondria and activation therein of Ca^{2+}-sensitive citrate cycle enzymes (Denton & McCormack, 1985) which are reflected in effects of the ion on oxidative phosphorylation (McCormack & Denton, 1993), indicate changes in this cell location by approx. 20s (Table 1).

The second major point revealed is that glucagon has several actions on vasopressin- and phenylephrine-induced Ca^{2+} mobilisation in the perfused rat liver; it reduces the latency of onset of net Ca^{2+} efflux (cf. Figs.1a and 2a and Figs.3a and 4a); it attenuates the rate, magnitude and duration of net Ca^{2+} influx and thereby brings forward (by some 30s) the time at which net influx commences. The other action, of enhanced Ca^{2+} influx, is already well documented (reviewed in Bygrave & Benedetti, 1993). Glucagon was also found to reduce the latency of onset of oxygen consumption induced by each of the two Ca^{2+}-mobilising agonists (Table 1).

While our data do not provide clues as to which second messengers, known (Irvine, 1992) or postulated (Randriamampita & Tsien, 1993) might be involved in the modulation of plasma membrane Ca^{2+} fluxes in liver, they do provide meaningful information that would have to be accommodated in any mechanism(s) arising from studies on single cells proposed to account for overall hormone-induced Ca^{2+} mobilisation (influx and efflux) in this cell type.

Insights into hormone-modulated bile flow

Others (Hill et al., 1985; Krell et al., 1985; Tsukada and Phillips, 1988; Hardison et al., 1991; Nathanson et al., 1992;) and ourselves (Hamada et al., 1992a,b) had recognised earlier that vasopressin or α_1-adrenergic agonists alone could induce rapid responses in bile flow (the first time point usually was not less than 60s following agonist administration). The 5s sampling intervals (Fig.1b) reveal an onset of vasopressin-induced flow by approx. 25s. Of added interest was the finding that prior administration of glucagon (Fig.2b) reduces the time of onset of vasopressin-induced bile flow to between 10 and 15s, and enhances the rate and magnitude of bile flow. Thus, even though a greater volume of bile has been extruded, the rate of outflow has returned to near basal values by the same time as with vasopressin alone (ie approx. 80s). Glucagon was seen also to significantly enhance the ability of phenylephrine to induce bile flow (cf. Figs.3b and 4b).

What we believe to be evident is that the role of Ca^{2+} in bile flow is more complex than previously appreciated. However it is pertinent that when one compares all of the information in Figs.1 to 4, it is apparent that a greater volume of bile is induced to flow under conditions where a smaller amount of Ca^{2+} effluxes from the liver. This and other related information from this study is summarised in Table 2.

Data in this chapter provides further evidence that cross-talk-mediated stimulation of Ca^{2+} fluxes in the perfused rat liver are reflected in the flow of bile, further implicating Ca^{2+} in bile flow events.

Table 2. Summary of information obtained in this study relating hormone-induced bile flow to changes in plasma membrane Ca^{2+} fluxes.

The information presented below was obtained from analyses of the data presented in Figs.1 to 4.

Agonist used	Bile flow[1]	Ca^{2+} mobilised		
		Efflux integral[2]	Max. efflux rate[3]	Max. influx rate[4]
Phenylephrine	0.19	135.5*	148	0.00
Vasopressin	0.39	34.7	82	85
Phenylephrine plus glucagon	0.59	18.7	56	212
Vasopressin plus glucagon	1.12	4.8	30	236

[1] µl of bile extruded/g of liver (data were obtained by integrating the area above the basal values in Figs.1b to 4b).

[2] nmol of Ca^{2+} released/g of liver (data were obtained by integrating the area above the base line in Figs.1a to 4a).

[3] nmol of Ca^{2+} efflux/min/g of liver in Figs.1a to 4a.

[4] nmol of Ca^{2+} influx/min/g of liver in Figs.1a to 4a.

* the value obtained applies to only the initial 100s of phenylephrine stimulation (see Fig.3a).

Chapter 2

Nickel: an agent for investigating the relation between hormone-induced Ca^{2+} influx and bile flow in the perfused rat liver

Nickel: an agent for investigating the relation between hormone-induced Ca^{2+} influx and bile flow in the perfused rat liver

INTRODUCTION

When liver cells are at rest, continuous cycling of calcium ions (Ca^{2+}) occurs particularly across the plasma membrane (see e.g. Bygrave, 1978; Reinhart et al., 1984a) ensuring maintenance of a relatively constant cytoplasmic Ca^{2+} concentration. Increases in cytoplasmic Ca^{2+} can occur rapidly in these and many other cell types upon stimulation by an appropriate agonist (Barritt, 1992). These increases in turn are crucial to a range of physiological responses including secretory events (e.g. Burgoyne and Morgan, 1993).

A major function of hepatocytes is the secretion of bile and the importance of extracellular Ca^{2+} in maintaining basal bile flow is well established. In this regard various groups (Graf, 1975; Owen, 1977) have found that a minimum extracellular Ca^{2+} concentration of 100 to 200μM is required. This requirement is thought to be related to the role of the ion in maintaining tight-junction integrity, rather than to the depletion of intracellular Ca^{2+} (Hardison, 1993, Nathanson et al., 1992a).

Ca^{2+}-mobilising hormones, known to rapidly induce increases in cytoplasmic Ca^{2+} in hepatocytes, also rapidly induce large changes in bile flow (e.g. Nathanson et al., 1992a,b; Hamada et al., 1992a,b; Karjalainen and Bygrave, 1994; reviews in Nathanson and Boyer, 1991; Bygrave et al., 1994). However the purported link between each of these complex events is presently unclear. In earlier work, Nathanson et al. (1992) concluded that the vasopressin-induced increase in intracellular Ca^{2+} attenuates bile flow, an effect diminished in the presence of 25μM Ni^{2+}. In consonance with data in the previous chapter this provided evidence for a correlative link between plasma membrane Ca^{2+} fluxes and bile flow following hormone stimulation of the perfused rat liver (Chapter 1).

In this chapter the above-mentioned observations have been extended by attenuating Ca^{2+} influx induced by Ca^{2+}-mobilizing hormones in the perfused liver with increasing concentrations of Ni^{2+} to progressively block such influx (Hughes and Barritt, 1989). The data from these experiments provide evidence for a multiple role for cytoplasmic Ca^{2+} in the mechanism of hormone-induced bile flow.

EXPERIMENTAL

The experimental procedures utilised in this section are essentially as set out in the General Methods section. Changes in perfusate Ca^{2+} were recorded by a Maclab recorder, based on a sample speed of 20 samples per s. Data were then analysed, calculated and graphed using IGOR (Wavemetrics, Oregon, U.S.A) software. Further details are contained in the legends to the figures.

RESULTS

Data in Figs.1 and 2 show results from a number of experiments wherein rat livers were perfused over a period and subjected to the synergistic action of vasopressin plus glucagon in the presence of increasing concentrations of Ni^{2+}. At the same time concomitant measurements of the perfusate Ca^{2+} concentration (Figs.1a, 2a) and bile flow (Figs.1b-f, 2b-d) were made. Aspects of these data are described.

(a) Immediate responses to Ni^{2+} addition

- changes in perfusate Ca^{2+}

An apparent efflux of the ion occurs immediately following Ni^{2+} addition. Control experiments indicated however that this response could be partly explained by an artefact due to the interaction of Ni^{2+} with the calcium electrode. On the other hand there are clear indications for a transient Ni^{2+}-induced stimulation of Ca^{2+} influx as reflected by the downward deflection of the trace. This is consistent with the conclusions of Beuers et al., (1993) that Ni^{2+} itself induces an increase in cytoplasmic Ca^{2+}, and with the observations in this work (data not shown) of increases in glucose output and oxygen uptake immediately following Ni^{2+} addition.

Fig.1. Effect of increasing concentrations of added Ni^{2+} on hormone-induced changes in perfusate Ca^{2+} and bile flow in the perfused rat liver.

Livers were perfused with Krebs-Henseleit bicarbonate medium containing 1.3mM Ca^{2+}. After a pre-perfusion period of 30min, glucagon and vasopressin (each at 10nM) were infused at the times indicated in the panels. Increasing concentrations of Ni^{2+} were present as follows: Fig.1b, control; Fig.1c, 25μM; Fig.1d, 100μM; Fig.1e, 250μM; Fig.1f, 500μM. Data in Fig.1a shows the average of at least 3 independent Ca^{2+} traces which were analysed as indicated in the Experimental section. For clarity traces prior to the addition of the hormones obtained for 25 and 100μM Ni^{2+} are not shown. Their patterns however were between those traces represented by the control and 250μM Ni^{2+}. In Fig.1a the letters represent the times at which A, Ni^{2+} was added; B, 10nM glucagon was added; C, 10nM vasopressin was added; D, Ni^{2+} infusion was terminated. For data on bile flow, the averages $\pm$ SEM from at least 8 independent experiments are shown.

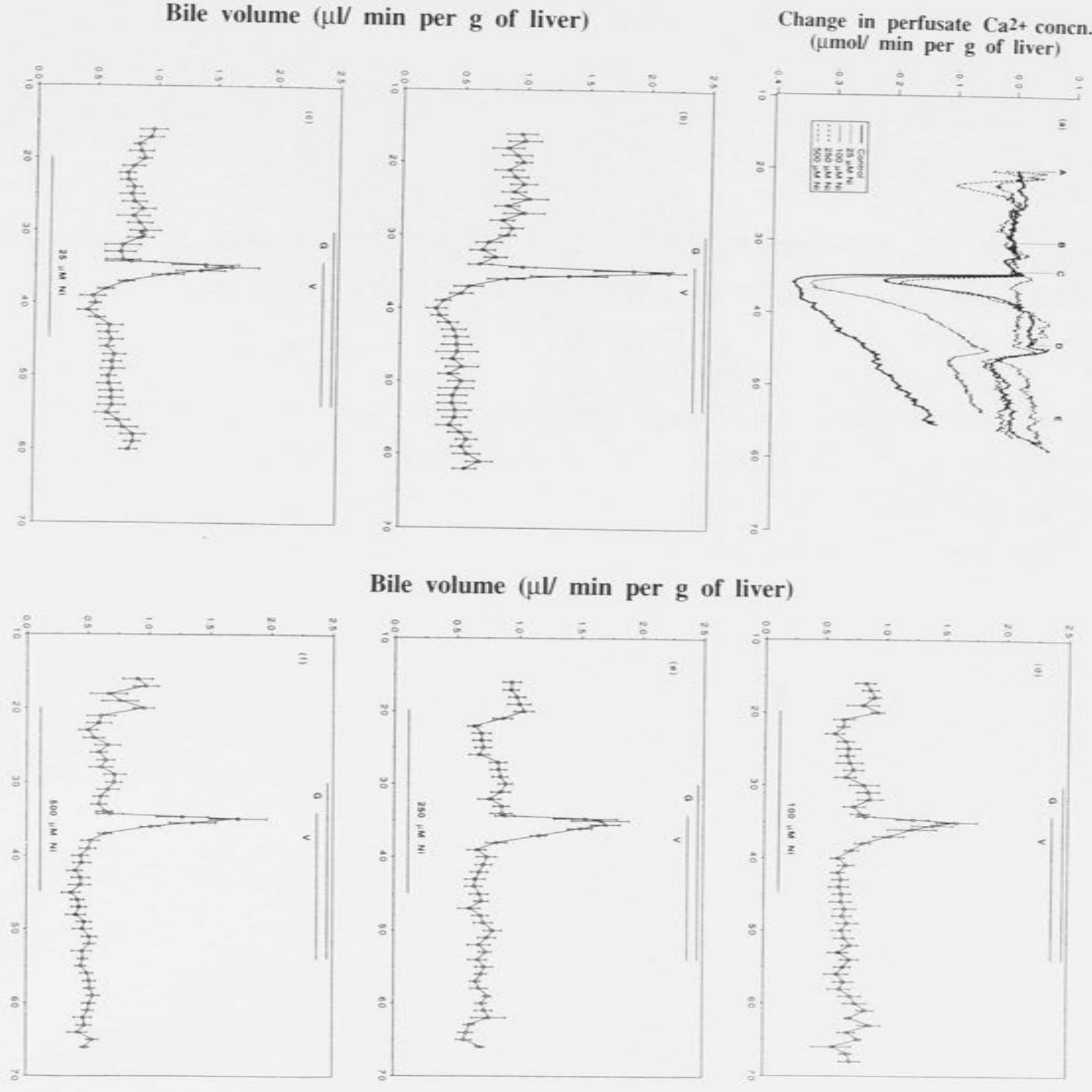
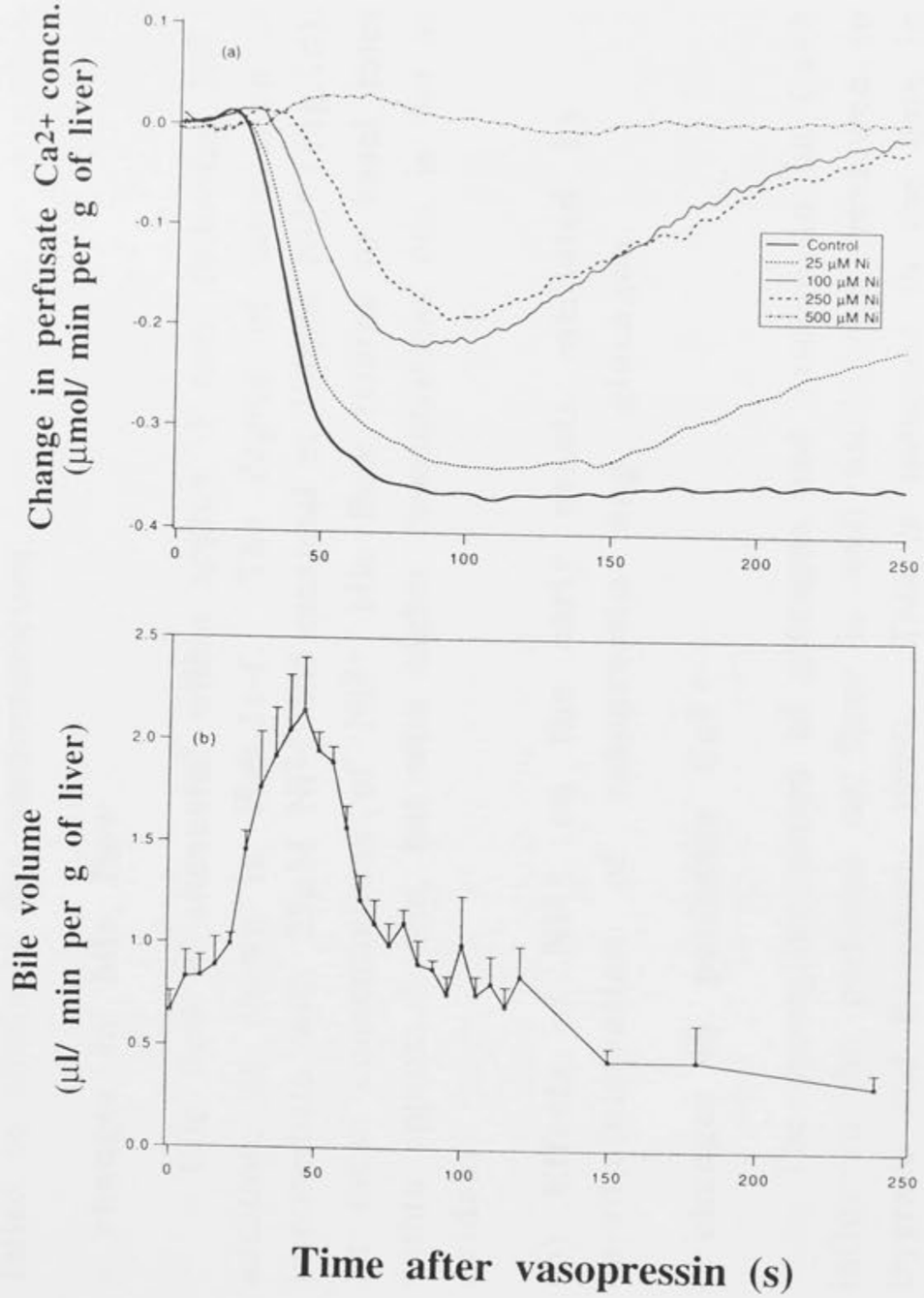
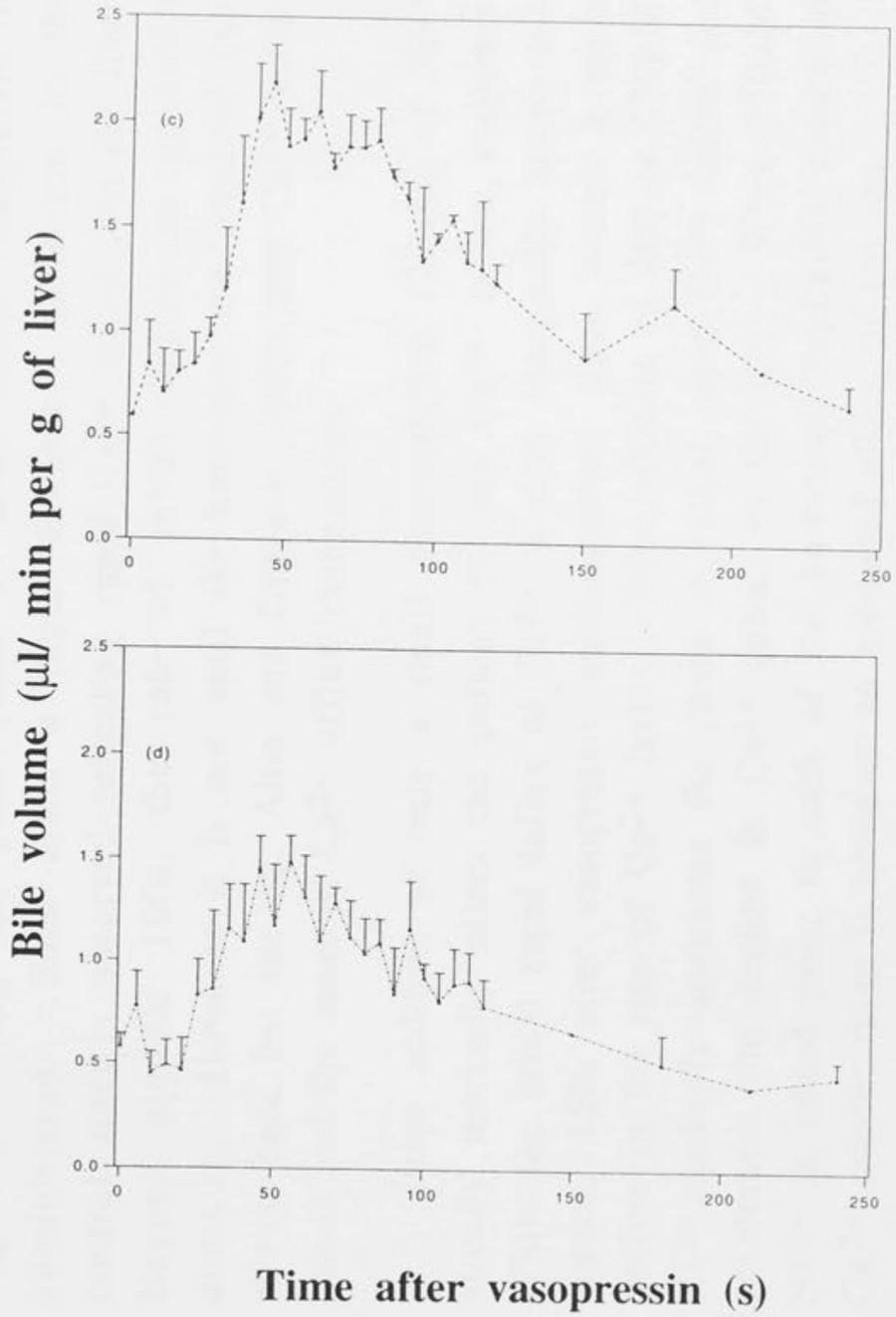

Change in perfusate Ca2+ concn. (μmol/ min per g of liver)
Control
25 μM Ni
100 μM Ni
250 μM Ni
500 μM Ni
A
B
C
D
E
Bile volume (μl/ min per g of liver)
25 μM Ni
100 μM Ni
250 μM Ni
500 μM Ni
G
V
Perfusion time (min)

Fig.2. Rapid responses of perfusate Ca^{2+} and bile flow to the synergistic action of glucagon and vasopressin in the perfused rat liver in the presence of increasing concentrations of added Ni^{2+}.

Data outlining the early rapid responses following administration of vasopressin in the presence of glucagon at time 34min in Fig.1 are shown in greater detail. Fig.2a shows the Ca^{2+} trace data for the various concentrations of added Ni^{2+}, Fig.2b shows bile data for the control, Fig.2c shows bile data with 250μM Ni^{2+} and Fig.2d shows bile data with 500μM Ni^{2+}. The average ± SEM from at least 3 independent experiments is shown.


(a)
(b)
(c)
(d)
Control
25 µM Ni
100 µM Ni
250 µM Ni
500 µM Ni
Change in perfusate Ca2+ concn. (µmol/ min per g of liver)
Bile volume (µl/ min per g of liver)
Time after vasopressin (s)


(c)
(d)
Bile volume (µl/ min per g of liver)
Time after vasopressin (s)

Although this influx was generally accompanied by vasoconstriction, as revealed by an increase in portal pressure, infusion of 200μM nitroprusside at the same time as 250μM Ni^{2+} failed to eliminate this vasoconstriction.

- changes in bile flow

Bile flow is attenuated within approx. 1 min. following Ni^{2+} addition, as shown in Figs.1c-f. The degree of attenuation is intermediate with 25μM Ni^{2+} and maximal at 100μM Ni^{2+} (Fig.1d). At these concentrations of Ni^{2+} bile flow returns to basal rates within approx. 5 min. but with higher concentrations this is not as evident.

(b) Effects of Ni^{2+} on the early events mediated by co-administration of vasopressin and glucagon.

- changes in perfusate Ca^{2+}

The synergistic action of glucagon and vasopressin on Ca^{2+} influx in the perfused rat liver is well established (reviewed in Bygrave and Benedetti, 1993). This is indicated in the data in Fig.1a following the point (time 34 min) where vasopressin is administered. Soon after, a rapid downward deflection in the control trace is observed reflecting rapid Ca^{2+} influx into the liver. Beyond approx. 100s, the rate of influx begins to gradually diminish. However it is not until the hormones are removed (not shown here but see eg. Altin and Bygrave, 1986) that Ca^{2+} influx ceases and the onset of Ca^{2+} efflux commences.

Prior addition of only a small concentration (25μM) of Ni^{2+} already markedly alters the pattern of this Ca^{2+} influx response. While the initial rapid influx of Ca^{2+} is only marginally attenuated, approx. 150s after vasopressin administration there occurs a rapid decline in the rate of Ca^{2+} influx. Prior addition of 100 or 250μM Ni^{2+} markedly attenuates the phase of initial rapid Ca^{2+} influx and accelerates the decline in Ca^{2+} uptake by the liver. When 500μM Ni^{2+} is added prior to each of the hormones, negligible uptake of Ca^{2+} results; there is however an enhanced efflux of the ion.

Data in Fig.2a are identical to that in Fig.1a except that they reveal (at a greater resolution) more clearly the early changes in

Ca^{2+} influx induced by the co-administration of the two hormones in the presence of various concentrations of added Ni^{2+}. In addition to what already has been described above, they show that as well as attenuating Ca^{2+} influx, increasing concentrations prolong the vasopressin-induced onset of Ca^{2+} influx. In the situation for example where 250μM Ni^{2+} is present, the onset of Ca^{2+} influx occurs some 15-20s later than that of the control.

- changes in bile flow

The following general trends are evident in the data in Fig.1. As routinely observed (Hamada et al., 1992a,b) and seen in Fig.1b, an early response to glucagon infusion in control liver is an immediate and transient attenuation of bile flow. As the Ni^{2+} concentration in the perfusate is increased to 250μM (Figs.1c-e) this glucagon-induced attenuation diminishes ($P < 0.01$ vs control, unpaired t-test). A second general trend is that as the Ni^{2+} concentration is increased to 250μM, there is a broadening of this peak. A third general trend is that whereas in control liver (Fig.1b) vasopressin induces an extremely rapid (sharp) reduction in bile flow to 0.32μl/min per g of liver, with a subsequent slight but significant ($p < 0.001$) recovery to 0.45μl/min per g of liver, increasing concentrations of Ni^{2+} up to 250μM counter this decrease and appear in fact to substantially increase the rate of subsequent bile flow. In fact, with 25μM Ni^{2+}, while the bile flow rate immediately following the peak is intermediate between control and 250μM added Ni^{2+}, there is a significant increase between 41 and 43 min, from 0.46 to 0.63μl/ min per g of liver ($p < 0.0001$). In both cases, there occurs a corresponding decrease in the Ca^{2+} influx rate.

The basic changes to the pattern of hormone-induced bile flow brought about by the prior addition of increasing Ni^{2+} concentrations to the perfusate, shown in Fig.1c-f, are reproduced at higher resolution for clarity in Figs.2b-d together with a higher resolution of the changes in perfusate Ca^{2+}. Similar to information in Chapter 1, these analyses show the very early events in both bile flow (made possible by 5s sampling of bile outflow) which can be directly correlated with Ca^{2+} flux changes. Approx. 20s after vasopressin administration to control liver (Fig.2b), bile flow rapidly increases to reach a maximum (bile flow rate approx. 2μl

per min per g of liver) by about 40s and thereafter rapidly declines to basal values by about 90s. In the presence of 250μM Ni^{2+}, where Ca^{2+} inflow is markedly attenuated (see Fig.2a), the initial phase of bile flow differs from that of the control in that the onset occurs 5 to 10s later (at approx. 30s) although the peak height is similar in magnitude. By contrast with the control, the decline in bile flow is considerably slower not reaching basal values until some 4 min after vasopressin infusion (Fig.2d); at 90s it still is approx. 50% of that seen at the peak. A similar pattern in bile flow is seen with 500μM Ni^{2+} (Fig.2e) but with a peak height less than that of the control and a return to basal values of bile flow by about 150s. Thus in this latter situation, significant outflow of bile at this phase is registered despite the absence of Ca^{2+} influx. While not shown here (but see data in Fig.1c,d and Table) we observed that the pattern of bile flow induced by glucagon plus vasopressin in the presence of 25 and 100μM Ni^{2+} was between that of the control (Fig.2b) and 250μM Ni^{2+} (Fig.2c).

(c) Immediate responses to termination of Ni^{2+} infusion - changes in perfusate Ca^{2+}

Termination of Ni^{2+} infusion at 45 min of perfusion (Fig.1a) with all concentrations employed led to a spontaneous upward and downward deflection in the Ca^{2+} trace. No significant effect on bile flow was detected at this point with any of the concentrations of Ni^{2+} employed (Fig.1b-f).

In other experiments (data not shown) we observed that addition of 500μM Ni^{2+} to the perfused rat liver soon after the administration of glucagon and vasopressin induced a rapid reversal of Ca^{2+} influx that quickly reached basal levels.

CONCLUSIONS

The present study confirms the report of Nathanson et al. (1992a) that Ni^{2+} is an effective inhibitor of Ca^{2+} influx in the perfused rat liver as it is in hepatocytes (Hughes and Barritt, 1989). Additionally, these data show that near-maximal inhibition of Ca^{2+} influx occurs at 500μM Ni^{2+} with half-maximal inhibition at approx. 200μM (Table); ie. values similar to those seen in hepatocytes where Ca^{2+} influx was assessed by quite different techniques (Hughes and Barritt, 1989). These data also reveal that half-

Table Responses to increasing concentrations of Ni^{2+} of the various parameters measured in the perfused rat liver that relate to Ca^{2+} fluxes and bile flow induced by the synergistic action of glucagon and vasopressin

The information below was derived from analyses of the data presented in Figs.1 and 2: bile flow, µl of bile extruded/g of liver (data obtained from integrating the area above basal values in Fig.1); amount released, nmol of Ca^{2+} released/g of liver (data obtained from integrating the area above the baseline in Fig.1a); max. efflux rate, nmol of Ca^{2+} efflux/min per g of liver in Fig.2a; max. influx rate, nmol of Ca^{2+} influx/min per g of liver in Fig.1; amount of Ca^{2+} taken up, nmol/g of liver

		Ca^{2+} mobilised			
$[Ni^{2+}]$ (mM)	Bile flow	Amount released	Max. efflux rate	Max. influx rate	Amount taken up
Control	1.6	2.3	14	370	5255
25	2.1	2.0	10	339	2538
100	2.4	4.8	15	220	418
250	2.7	2.3	16	193	372
500	1.5	23	31	0	0

maximal inhibition of the total amount of Ca^{2+} taken up by the liver under these conditions occurs at Ni^{2+} concentrations of less than 25μM (Table). Besides inhibiting Ca^{2+} influx, Ni^{2+} also delayed the onset of both Ca^{2+} influx and efflux.

The effects of Ni^{2+} on bile flow in the perfused rat liver are clearly complex; each of the three phases modulated by the actions of glucagon plus vasopressin (Bygrave et al., 1994) were significantly altered by the prior addition of Ni^{2+} to the perfusion medium. The first of these was the attenuation by increasing concentrations of Ni^{2+} of glucagon-induced transient cholestasis (cf. the 30-34 min perfusion time in Fig.1b with that in Fig.1e, for example). The second phase, whereby vasopressin rapidly enhances bile flow, was modified and the third whereby a subsequent action of vasopressin was to rapidly induce a marked cholestasis, was also offset. We found in addition that bile flow responses were delayed by a time interval concomitant with the above-mentioned delay in Ca^{2+} influx though this delay was difficult to quantitate.

Further evidence for the functional link between Ca^{2+} inflow and induced bile flow is indicated from experiments where 250μM Ni^{2+} was used; here the initial transient response is prolonged at a much higher rate. The hormone-induced stimulation of a relatively large but brief influx in the presence of 250μM Ni^{2+}, therefore may allow maximization of the stimulatory and minimization of the inhibitory effects of intracellular Ca^{2+} increases. Thus the data indicate that both the rate and the duration of Ca^{2+} influx is important in determining the amount of bile released under these conditions. The importance of this Ca^{2+} influx rate on bile flow is further suggested by the increase in bile flow that occurs following the vasopressin-induced nadir (41-43 min in Fig.1c) which is seen during hormone stimulation with both the control liver and following the addition of 25μM Ni^{2+}; this appears to correspond to a decrease in the Ca^{2+} influx rate at these points.

Conditions where Ca^{2+} is derived from intracellular stores alone, as seen for example with 500μM Ni^{2+} (which induces Ca^{2+} efflux only) or with phenylephrine or the initial efflux phase induced by vasopressin alone (Chapter 1), may not be sufficient to trigger the response as sharply as seen with the control and with

250μM Ni^{2+} present (Fig.2). However, the amount of bile flow in the presence of 500μM Ni^{2+}, while less than with 250μM Ni^{2+}, is much greater than that seen in the presence of vasopressin alone (0.39μl/g of liver, (Chapter 1)). This could be explained by the counteraction of the inhibitory actions of Ca^{2+} by Ni^{2+} as well as by the choleretic effects of glucagon or its second messenger cyclic AMP (Hayakawa et al., 1990). It is noteworthy that basal bile flow is sustained at a significantly lower rate in the presence of this high concentration of Ni^{2+} (Fig.1f). At the same time some of the data are consistent with depletion of intracellular Ca^{2+} stores

The data thus reflect the complexity of the relationship between Ca^{2+} fluxes and bile flow, and the possible importance of the localization of the intracellular Ca^{2+} in these events.

Chapter 3

Modulation of Ca^{2+} fluxes and bile flow in the perfused rat liver by cross-talk between sub-maximal concentrations of vasopressin and glucagon

Modulation of Ca^{2+} fluxes and bile flow in the perfused rat liver by cross-talk between sub-maximal concentrations of vasopressin and glucagon

INTRODUCTION

In the phenomenon of signalling cross-talk, individual signal-transducing systems interact to produce a modulated response different from that produced by the individual systems. This concept is gaining prominence as having an important role in regulatory events in cells of many tissues. The molecular mechanisms of such cross-talk are still unclear but may well involve interactions at the level of the receptors themselves, of G-proteins and/or of the second messengers generated (recently reviewed by Bygrave and Roberts, 1995).

One tissue in which the phenomenon of cross-talk is particularly evident is liver. Here the interactions resulting from the combined exposure of either isolated cells or the whole organ to glucagon (which acts mainly through the generation of cyclic AMP) and Ca^{2+}-mobilising agonists like vasopressin (acting through IP_3 generation), result in changes in Ca^{2+} fluxes that are considerably different from those obtained with either hormone alone (reviewed in Altin and Bygrave, 1988 and Bygrave and Benedetti, 1993). These interaction(s) manifest themselves in the fine regulation, not only of cellular Ca^{2+} fluxes, but also of important Ca^{2+}-sensitive physiological responses such as bile flow (Hamada et al., 1992a,b; reviewed in Bygrave et al., 1994; Chapter 1).

In most studies where cross-talk has been examined in liver, the concentrations of agonists used generally have been supra-maximal. The use of such concentrations of agonists however could have masked other subtle responses that might occur following the actions of these agonists when used at sub-maximal concentrations. This prompted an exploration of cross-talk in the perfused rat liver using vasopressin and glucagon separately and in combination over a wider range of concentrations than previously adopted. The perfused liver technique allows one to examine the onset and progress of both net Ca^{2+} inflow and net Ca^{2+} outflow. This

provides for further insights into the hormone-induced Ca^{2+} flux changes, in particular the inter-dependence or otherwise of the efflux of Ca^{2+} released from the intracellular (endoplasmic reticulum) store and influx of Ca^{2+} across the plasma membrane. Additionally, concomitant analysis of bile flow enables examination of a physiological response to the role of cross-talk between the signalling systems induced by these agents on this parameter.

Data from experiments in this chapter have revealed at least two new important aspects about hormone-induced Ca^{2+} fluxes: (a) that when very low concentrations of vasopressin alone are used, it is possible to discriminate between effects on net Ca^{2+} inflow and those on net Ca^{2+} outflow, and (b) that glucagon is able to sensitise both Ca^{2+} efflux and influx under various conditions, the prominent effect being on the influx of Ca^{2+}. In addition, cross-talk between these signalling pathways profoundly increases the sensitivity of bile flow to modulation by hormones.

EXPERIMENTAL

The experimental procedures utilised in this section are essentially as set out in the General Methods section. Changes in perfusate Ca^{2+} were recorded by a Maclab recorder, based on a sample speed of 20 samples per s. Data were then analysed, calculated and graphed using IGOR (Wavemetrics, Oregon, U.SA) software. Further details are contained in the legends to the figures.

RESULTS

In previous work from this laboratory (Altin and Bygrave, 1986) the agonist concentration dependence of the maximal rate of Ca^{2+} influx in the synergistic mobilisation of Ca^{2+} in the perfused rat liver was examined. In these experiments, the concentration of one agent was maintained at a concentration which resulted in a maximal response (10nM), while the concentration of the other was varied. In the present study the effect of administering both glucagon and vasopressin over a range of low concentrations has been examined.

Ca^{2+} fluxes in the perfused rat liver induced by low concentrations of vasopressin alone

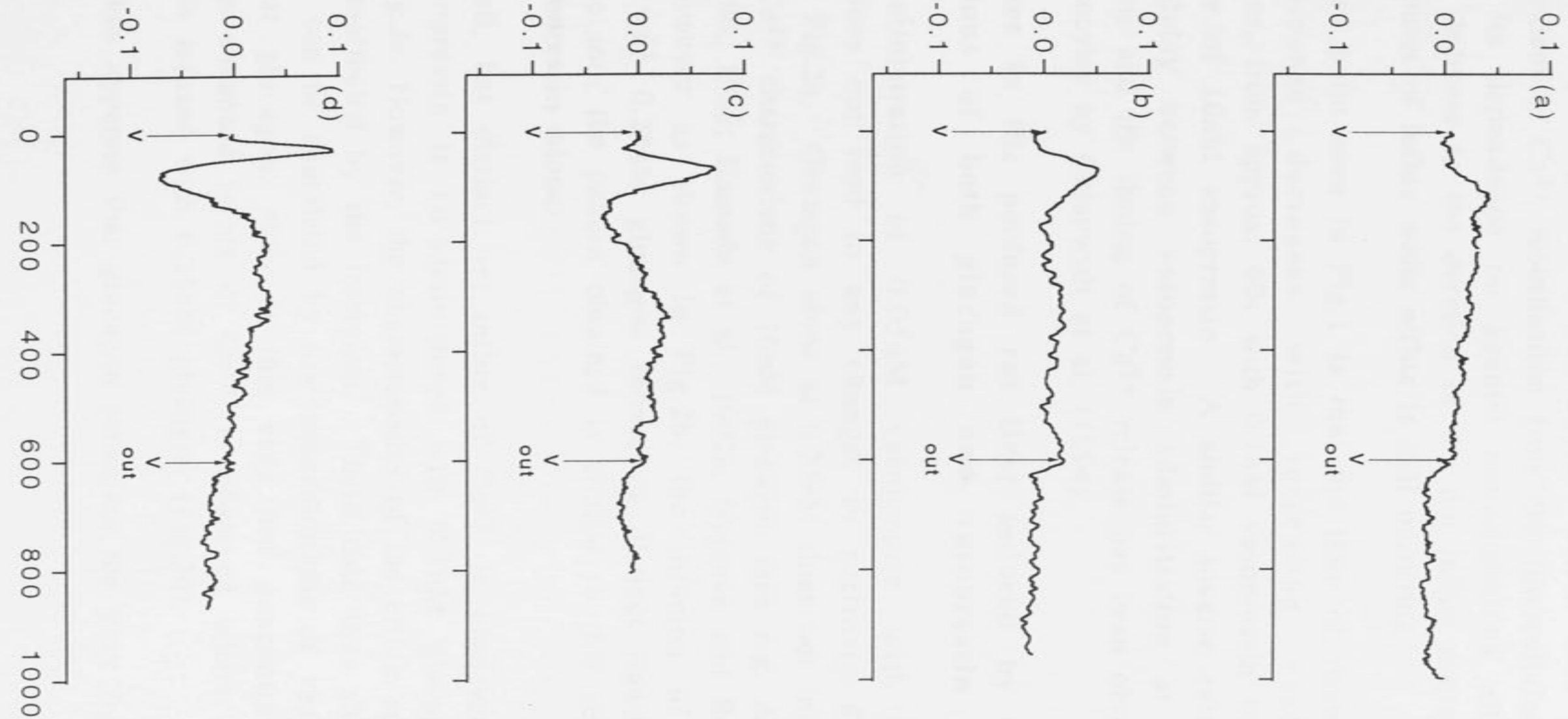
Data in Fig.1 show results from representative experiments in which vasopressin alone was infused from time zero to 600s. The lowest concentration of vasopressin at which a response in Ca^{2+} fluxes was observed occurred at 0.1nM (Fig.1a). In other experiments (data not shown) no Ca^{2+} flux changes were observed with 0.05nM vasopressin. While in these experiments 0.1nM vasopressin appeared to be at the lower limit of the sensitivity of the experimental system, at 0.25nM vasopressin alone (Fig.1b) a transient efflux of Ca^{2+} is consistently observed. No net influx takes place until administration of the hormone is terminated at 600s. At 1nM vasopressin a small net influx is observed (Fig.1c), and at 5nM (data not shown) the Ca^{2+} flux response begins to reflect that consistently observed at 10nM vasopressin (Fig.1d). This latter concentration normally is used in many other experiments including those with hepatocyte preparations and with single hepatocytes. This pattern is characterised by a net efflux of the ion followed spontaneously by a net influx (Fig.1d) (see also eg. Altin and Bygrave, 1986; Hamada et al., 1992a). These data thus indicate that a threshold vasopressin concentration of approximately 0.1nM exists, above which induction of net efflux occurs, and that net influx is induced only at concentrations of vasopressin greater than approximately 1nM. Also, where vasopressin infusion results in efflux alone, influx of Ca^{2+} occurs only upon removal of the hormone. This latter feature is reminiscent of the actions of a high concentration of phenylephrine (Altin and Bygrave, 1985).

As indicated in the Fig.2 of the Introductory Review , no change in the rate of glucose release is observed at 0.05nM vasopressin. Glucose release is seen at 0.1nM vasopressin however, and is maximal at approximately 1nM. These data suggest that in the perfused rat liver, as in isolated cells (see e.g. Thomas et al., 1984; Sanchez-Bueno et al., 1993), vasopressin at 0.1nM is just commencing to release Ca^{2+} from hormone-sensitive intracellular Ca^{2+} stores.

A second general trend apparent in Fig.1 is that the maximum efflux rate increases as the concentration of the agonist increases. In addition, the maximum rate of change in efflux rate is also

Fig.1. Changes in perfusate Ca^{2+} in response to infusing with varying low concentrations of vasopressin.

Livers were perfused with Krebs-Henseleit bicarbonate medium containing 1.3mM Ca^{2+}. After a pre-perfusion period of 30min, vasopressin was infused at a concentration of (a) 0.1nM, (b) 0.25nM, (c) 1.0nM and (d) 10.0nM from zero to 600 seconds as indicated in the panels. Each trace is representative of at least three independent experiments. The data have been corrected for a lag of 11 ± 1 seconds, which is the time taken, in this experimental system, for Ca^{2+} to flow from the point of entry into the liver and then away from the liver to the Ca^{2+} electrode.


Change in perfusate Ca^{2+} concentration
(μmol/ min per g of liver)
0.1
0.0
-0.1
(a)
(b)
(c)
(d)
v
V
out
0
200
400
600
800
1000
Time after vasopressin (s)

proportional to the agonist concentration. These data probably reflect the extent of Ca^{2+} mobilization from the intracellular storage sites, and its dependence on agonist concentrations alluded to above. The decrease in the duration of the net influx response may reflect the onset of influx while efflux is still occurring.

A third point seen in Fig.1 is that the time of onset of the efflux response decreases with increasing vasopressin concentrations, from approx. 60s with 0.1nM vasopressin to 11s in the presence of 10nM vasopressin. A similar inverse relationship with the delay between vasopressin administration at various concentrations and the timing of Ca^{2+} release has been observed in single hepatocytes by Chiavaroli et al. (1994).

Ca^{2+} fluxes in the perfused rat liver induced by low concentrations of both glucagon and vasopressin

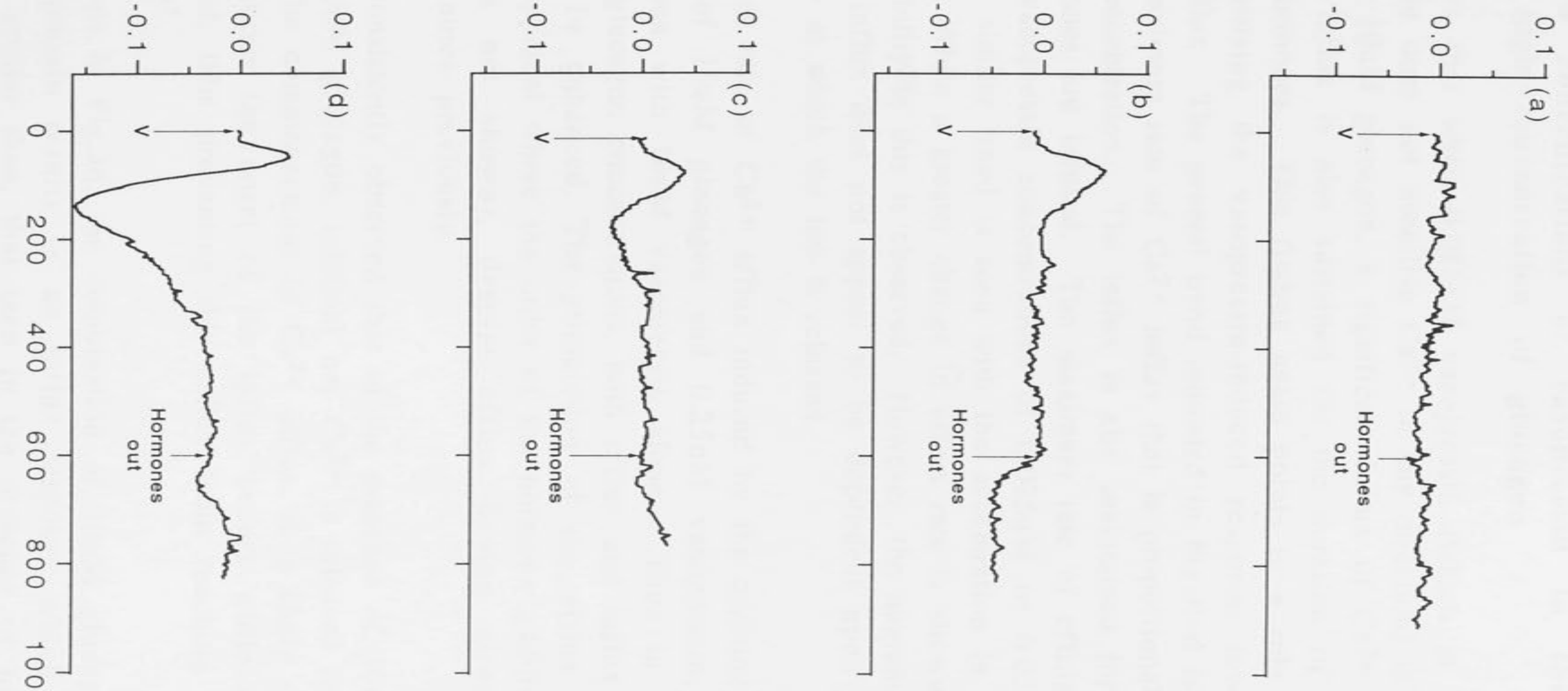
Co-administration of 0.05nM vasopressin with 0.25nM glucagon, does not lead to any changes in perfusate Ca^{2+}, as indicated in Fig.2a. Glucagon alone at 0.25nM does not induce the release of Ca^{2+} characteristic of 10nM glucagon (see e.g. Altin and Bygrave, 1986, 1988; Hamada et al., 1992a, Bygrave and Benedetti, 1993). However as shown in Fig.2b, the infusion of 0.1nM vasopressin with 0.25nM glucagon induces a distinct transient net efflux of the ion; the pattern obtained is similar to that seen with 0.25nM vasopressin alone.

A small, but distinct net influx of Ca^{2+} is observed when 0.25nM vasopressin is co-administered with 0.1nM glucagon, as shown in Fig.2c. However, the characteristics of the efflux appear to be largely unaffected by the treatment. These data thus show that Ca^{2+} influx can be stimulated by low concentrations of vasopressin provided that glucagon, even at this very low concentration, is present. An enhanced influx of Ca^{2+} is observed where 0.25nM vasopressin is infused with 0.25nM glucagon (Fig.2d).

It is thus apparent that glucagon sensitises the liver for influx of Ca^{2+}.

Fig.2. Changes in perfusate Ca^{2+} in response to infusing with varying low concentrations of glucagon and vasopressin.

The experimental system was the same as that described in Figure 1, except that glucagon was infused into the liver 4 min before, and then concomitant with, the administration of vasopressin from zero to 600 seconds. The regimes used were (a) 0.25nM glucagon and 0.05nM vasopressin, (b) 0.25nM glucagon and 0.1nM vasopressin, (c) 0.1nM glucagon and 0.25nM vasopressin and (d) 0.25nM glucagon and 0.25nM vasopressin.


Change in perfusate Ca^{2+} concentration
(μmol/ min per g of liver)
(a)
(b)
(c)
(d)
-0.1
0.0
0.1
v
Hormones
out
0
200
400
600
800
1000
Time after vasopressin (s)

Infusion of low concentrations of vasopressin in the presence of a high concentration of glucagon

Fig.3a shows that when 0.025nM vasopressin (which at this concentration alone does not mobilise Ca^{2+} in any direction) is co-administered with 10nM glucagon, a significant influx of Ca^{2+} can be induced; this influx is also sustained for the duration of the infusion of the hormones. This finding again points to a role for glucagon in sensitising the vasopressin-induced response toward inducing Ca^{2+} influx. The general trend indicated in Fig.3b-d is an increase in the maximum rate of Ca^{2+} influx that is proportional to the vasopressin concentration. The influx is also maintained for as long as the hormones are infused. The maximum rate of efflux is very low where vasopressin concentrations of 0.025nM or 0.05nM are employed. A similar trend is seen with the acceleration in the Ca^{2+} efflux rate. While a greater change in efflux rate is shown in Fig.3c, some variability in this is observed. However, the amount or the rate of Ca^{2+} influx does not appear to be dependent upon the amount or the rate at which the ion is released.

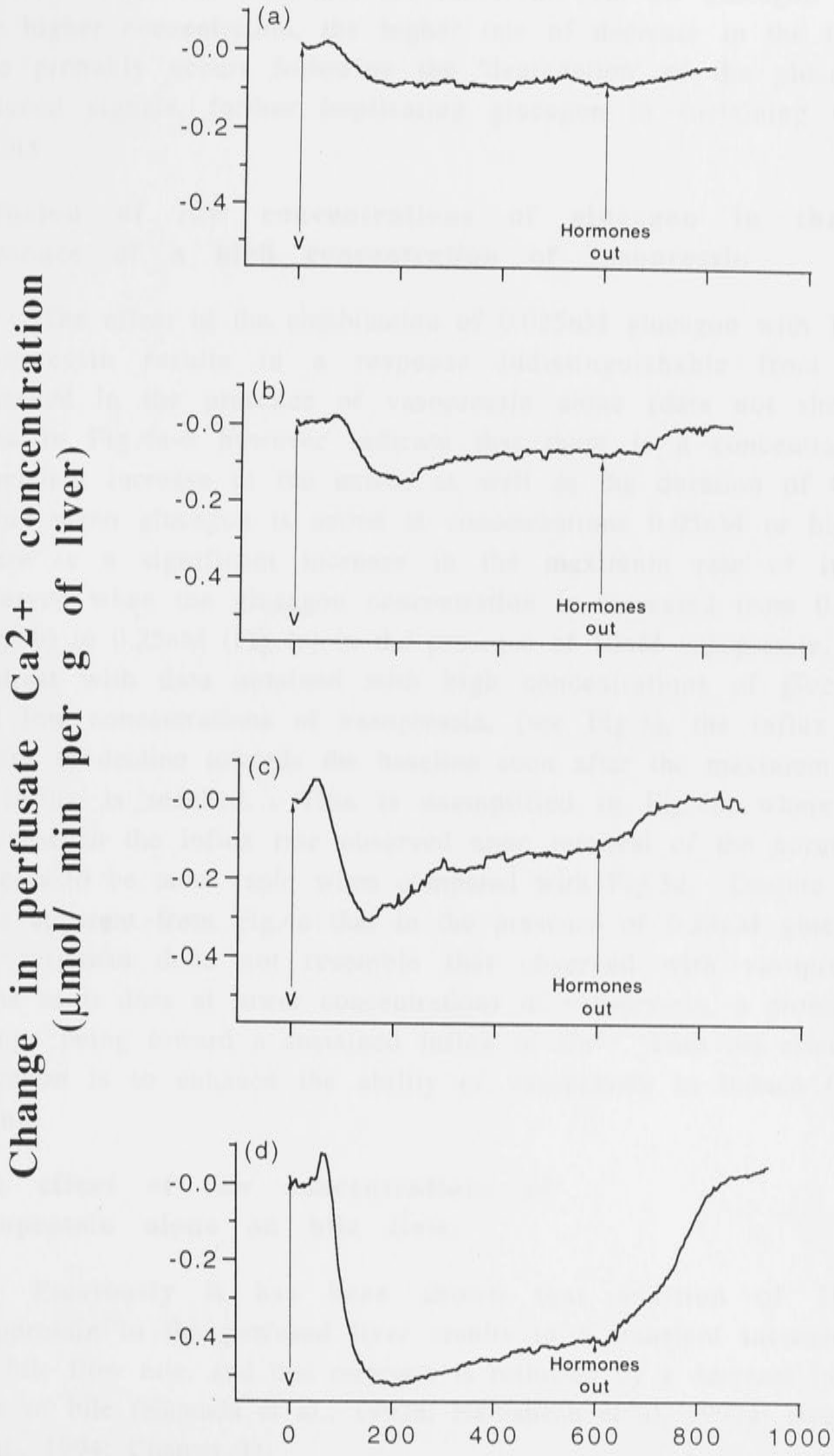
The maximum rate of Ca^{2+} efflux induced by the addition of the combination of 10nM glucagon and 0.25nM vasopressin, is similar to that seen with 10nM vasopressin alone. Thus in the presence of high glucagon concentrations, both efflux and influx of Ca^{2+} appear to be enhanced. The stimulation of the efflux by glucagon is also apparent where the order of the hormone additions is reversed (data not shown), despite efflux having already occurred some minutes previously.

Since it is consistently observed that in the presence of 10nM vasopressin and 10nM glucagon, minimal net Ca^{2+} is released from the cell prior to the commencement of Ca^{2+} influx, it is likely that under these conditions the onset of the influx occurs while the efflux is stimulated, thus preventing this efflux from reaching its maximum potential.

As is apparent in Fig.3d, the combination of 10nM glucagon and 0.25nM vasopressin results in an influx response which is similar to, if not greater than, that seen in the presence of high concentrations of both hormones. An additional feature is the biphasic nature of the decrease in the rate of influx, which is

Fig.3. Changes in perfusate Ca^{2+} in response to infusing with 10nM glucagon and varying low concentrations of vasopressin.

The experimental system was the same as that described in Figure 1, except that glucagon (10nM) was infused into the liver 4 min before, and then concomitant with, the administration of vasopressin at the following concentrations from zero time to 600 seconds: (a) 0.025nM, (b) 0.05nM, (c) 0.1nM and (d) 0.25nM.


(a)
-0.0
-0.2
-0.4
V
Hormones out
(b)
-0.0
-0.2
-0.4
V
Hormones out
(c)
-0.0
-0.2
-0.4
V
Hormones out
(d)
-0.0
-0.2
-0.4
V
Hormones out
0
200
400
600
800
1000
Change in perfusate Ca2+ concentration (μmol/ min per g of liver)
Time after vasopressin (s)

observed when the hormones are removed. As the glucagon is at the higher concentration, the higher rate of decrease in the influx rate probably occurs following the 'degradation' of the glucagon-induced signals, further implicating glucagon in sustaining Ca^{2+} influx.

Infusion of low concentrations of glucagon in the presence of a high concentration of vasopressin

The effect of the combination of 0.025nM glucagon with 10nM vasopressin results in a response indistinguishable from that observed in the presence of vasopressin alone (data not shown). Data in Fig.4a-d however indicate that there is a concentration-dependent increase in the extent as well as the duration of Ca^{2+} influx when glucagon is added at concentrations 0.05nM or higher. There is a significant increase in the maximum rate of influx observed when the glucagon concentration is increased from 0.1nM (Fig.4b) to 0.25nM (Fig.4c) in the presence of 10nM vasopressin. By contrast with data obtained with high concentrations of glucagon and low concentrations of vasopressin, (see Fig.3), the influx rate begins to decline towards the baseline soon after the maximum rate of influx is reached. This is exemplified in Fig.4c, where the decrease in the influx rate observed upon removal of the hormones appears to be more rapid when compared with Fig.3d. Despite this, it is apparent from Fig.4c that in the presence of 0.25nM glucagon the response does not resemble that observed with vasopressin alone as it does at lower concentrations of vasopressin, a prominent change being toward a sustained influx of Ca^{2+}. Thus the effect of glucagon is to enhance the ability of vasopressin to induce Ca^{2+} influx.

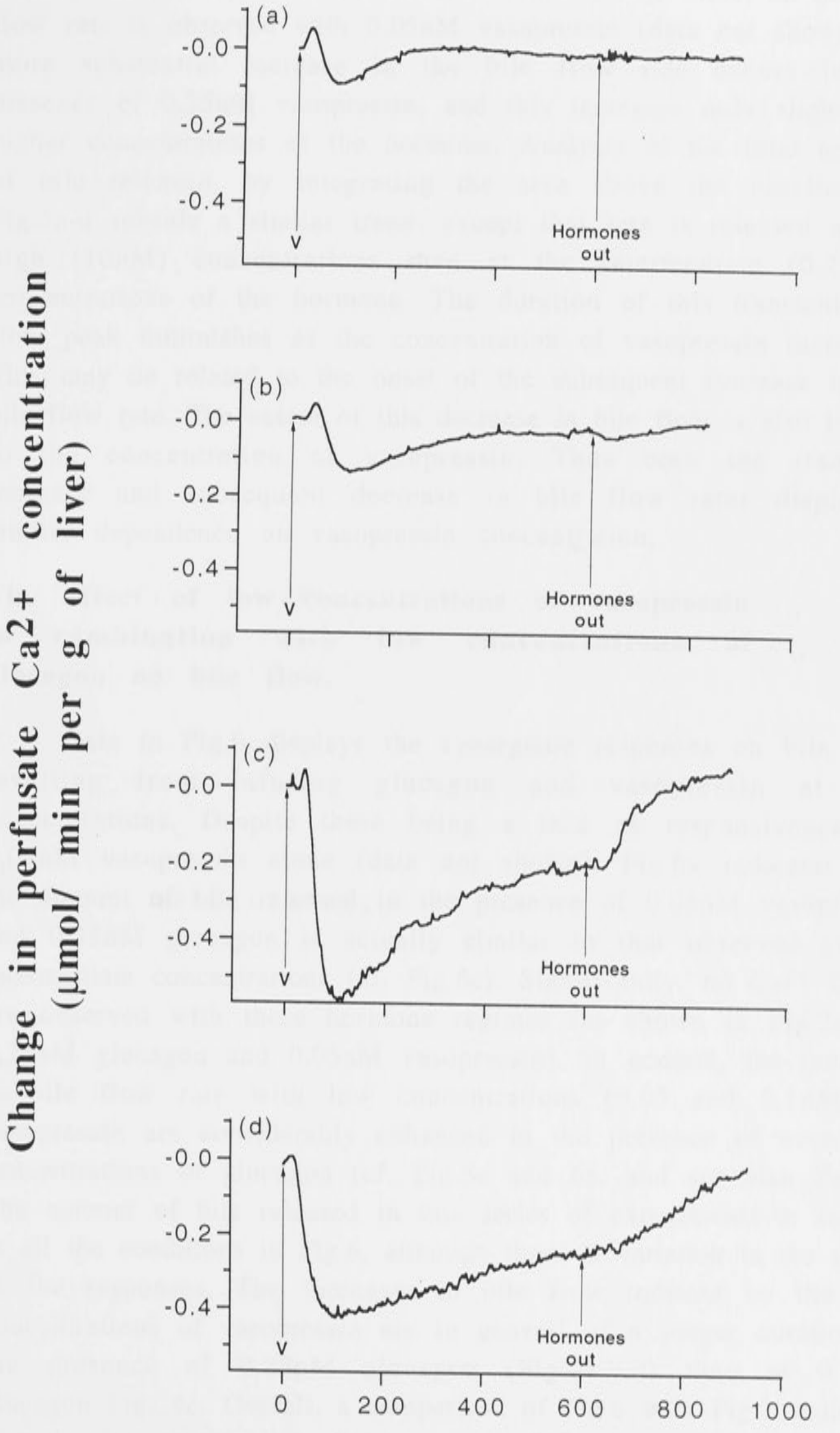
The effect of low concentrations of vasopressin alone on bile flow.

Previously it has been shown that addition of 10nM vasopressin to the perfused liver results in a transient increase in the bile flow rate, and this response is followed by a decrease in the flow of bile (Hamada et al., 1992a; Nathanson et al, 1992a; Bygrave et al., 1994; Chapter 1).

With 0.1nM vasopressin, a small increase in bile flow rate occurs, which is sustained for some minutes, when a significant

Fig.4. Changes in perfusate Ca^{2+} in response to infusing with varying low concentrations of glucagon and 10nM vasopressin.

The experimental system was the same as that described in Figure 1, except that glucagon was infused into the liver 4 min before, and then concomitant with, the administration of 10nM vasopressin from zero time to 600 seconds. Glucagon was infused at (a) 0.05nM, (b) 0.1nM, (c) 0.25nM and (d) 10.0nM.


(a)
-0.0
-0.2
-0.4
V
Hormones out
(b)
-0.0
-0.2
-0.4
V
Hormones out
(c)
-0.0
-0.2
-0.4
V
Hormones out
(d)
-0.0
-0.2
-0.4
V
Hormones out
0
200
400
600
800
1000
Change in perfusate Ca2+ concentration (μmol/ min per g of liver)
Time after vasopressin (s)

decrease in the bile flow rate occurs. However, no effect on the bile flow rate is observed with 0.05nM vasopressin (data not shown). A more substantial increase in the bile flow rate occurs in the presence of 0.25nM vasopressin, and this increases only slightly at higher concentrations of the hormone. Analysis of the total amount of bile released, by integrating the area above the baseline, in Fig.5a-d reveals a similar trend, except that less is released at the high (10nM) concentrations than at the intermediate (0.25nM) concentrations of the hormone. The duration of this transient bile flow peak diminishes as the concentration of vasopressin increases. This may be related to the onset of the subsequent decrease in the bile flow rate. The extent of this decrease in bile flow is also related to the concentration of vasopressin. Thus both the transient increase and subsequent decrease in bile flow rates display a similar dependence on vasopressin concentration.

The effect of low concentrations of vasopressin in combination with low concentrations of glucagon on bile flow.

Data in Fig.6 displays the synergistic responses on bile flow resulting from infusing glucagon and vasopressin at low concentrations. Despite there being a lack of responsiveness to 0.05nM vasopressin alone (data not shown), Fig.6a indicates that the amount of bile released in the presence of 0.05nM vasopressin and 0.25nM glucagon is actually similar to that observed at the intermediate concentrations (cf. Fig.6c). Significantly, no Ca^{2+} fluxes are observed with these hormone regimes (as shown in Fig.2a for 0.25nM glucagon and 0.05nM vasopressin). In general, the increase in bile flow rate with low concentrations (0.05 and 0.1nM) of vasopressin are considerably enhanced in the presence of even low concentrations of glucagon (cf. Fig.5a and 6a, and see also Table). The amount of bile released in this series of experiments is similar in all the conditions in Fig.6, although there is variation in the shape of the responses. The increases in bile flow induced by the low concentrations of vasopressin are in general of a longer duration in the presence of 0.25nM glucagon (Fig.6a,b,d) than at 0.1nM glucagon Fig. 4c. Overall, a comparison of Fig.6 with Fig.2 indicates that despite considerable differences in Ca^{2+} fluxes being observed with the combinations of low doses of the hormones, these are not reflected in the release of bile.

Fig.5. Changes in the bile flow rate in response to infusing with varying low concentrations of vasopressin.

The changes in bile flow rate were measured concomitantly with the changes in Ca^{2+} concentration shown in Figure 1, in response to the infusion of vasopressin at (a) 0.1nM, (b) 0.25nM, (c) 1.0nM and (d) 10.0nM from zero to 600 seconds. Means ± S.E.M. from three to five experiments are shown.

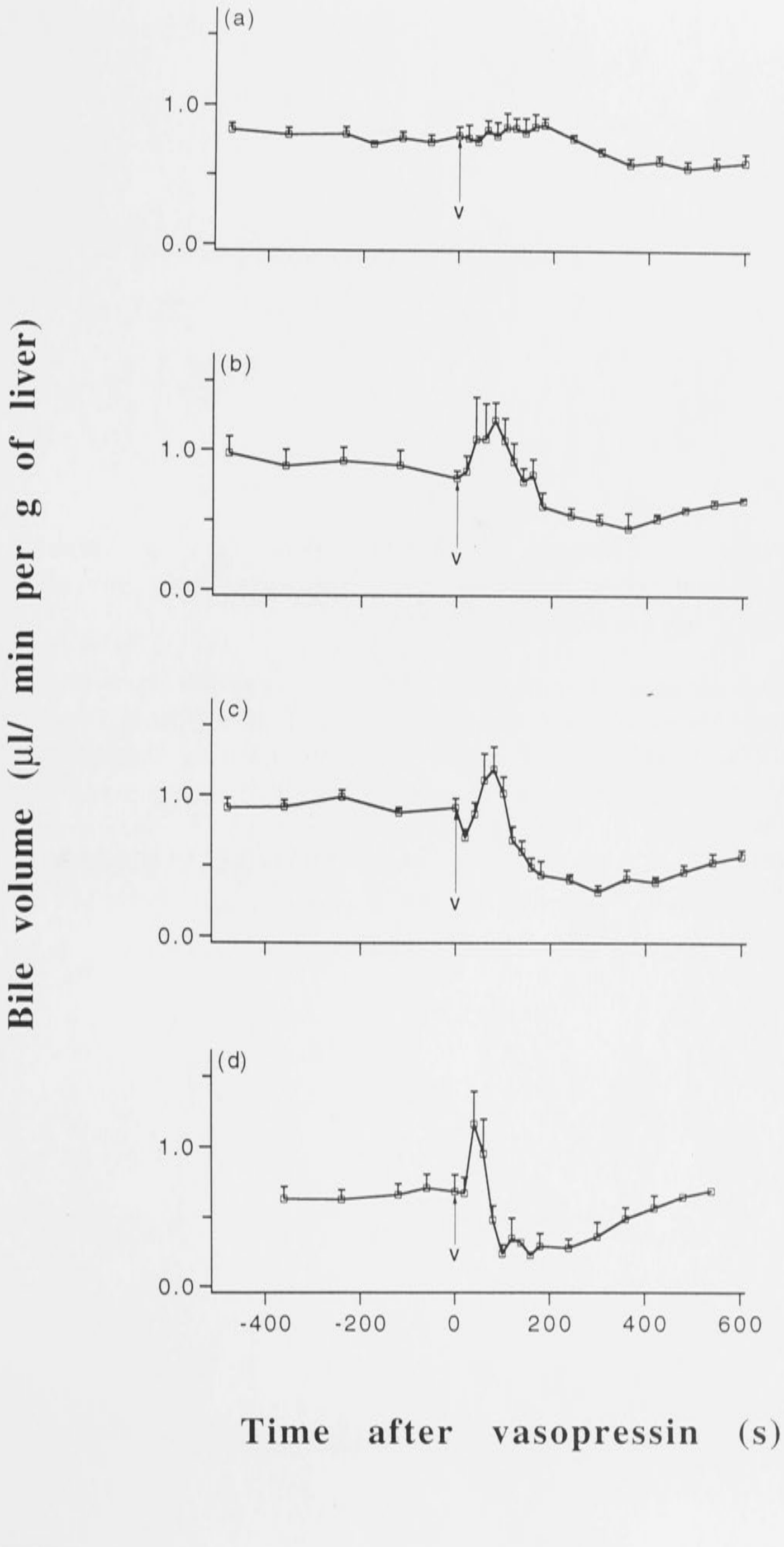

(a)
1.0
0.0
V
(b)
1.0
0.0
V
(c)
1.0
0.0
V
(d)
1.0
0.0
V
-400
-200
0
200
400
600
Bile volume (μl/ min per g of liver)
Time after vasopressin (s)

Fig.6. Changes in the bile flow rate in response to infusing with varying low concentrations of glucagon and vasopressin.

The changes in bile flow rate were measured concomitantly with the changes in Ca^{2+} concentration shown in Figure 2, i.e. in response to the infusion of hormones with the following regimes (a) 0.25nM glucagon and 0.05nM vasopressin, (b) 0.25nM glucagon and 0.1nM vasopressin, (c) 0.1nM glucagon and 0.25nM vasopressin and (d) 0.25nM glucagon and 0.25nM vasopressin, vasopressin being present from zero time to 600 seconds. Means ± S.E.M. from three to five experiments are shown.

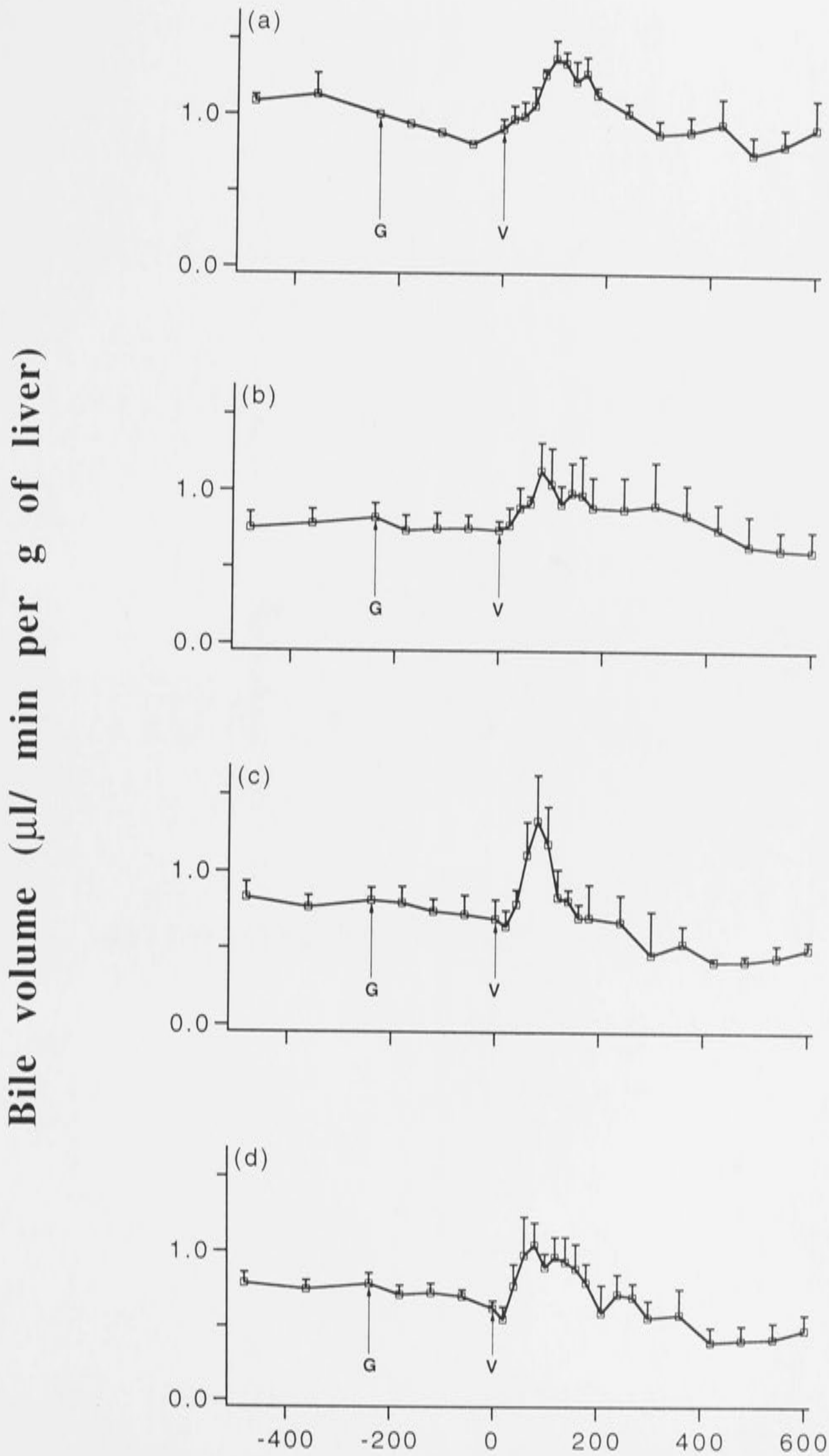

(a)
1.0
0.0
G
V
(b)
1.0
0.0
G
V
(c)
1.0
0.0
G
V
(d)
1.0
0.0
G
V
-400
-200
0
200
400
600
Bile volume (μl/ min per g of liver)
Time after vasopressin (s)

TABLE Summary of the effects of vasopressin and glucagon on early bile flow in the perfused rat liver.

The data were obtained by comparing the changes in bile flow against the baseline. The duration of a response refers to the time elapsed from the start of a response to the time when it returns to the baseline. The increase and decrease in bile flow rate refer to the average maximum and minimum rates in bile flow rate from the baseline respectively. The amount of bile released and the amount of deficit in bile flow were obtained by integrating the area above or below the baseline respectively. Note that in order to enable comparisons between all the experiments to be made, the amount of deficit in bile flow was only measured for 10min.

Summary of the effects of vasopressin and glucagon on early bile flow in the perfused rat liver.

Hormone treatment			Bile flow increase		Bile flow deficit	
[Glucagon] (nM)	[Vasopressin] (nM)	Duration (s)	Amount released (µl/g)	Increase in bile flow rate (µl/min/g)	Amount of deficit (µl/g)	Decrease in bile flow rate (µl/min/g)
	0.05	-	-	-	-	-
-	0.1	240	0.17	0.11	1.19	0.17
-	0.25	120	0.45	0.41	3.0	0.27
-	1.0	60	0.31	0.40	3.9	0.39
-	10.0	60	0.22	0.49	1.8	0.39
0.1	0.1	160	0.47	0.21	0.64	0.13
0.25	0.05	240	0.61	0.36	1.15	0.20
0.25	0.1	140	0.34	0.30	1.11	0.21
0.1	0.25	80	0.48	0.57	2.34	0.31
0.25	0.25	140	0.44	0.30	2.17	0.33
10	0.025	180	0.63	0.43	1.48	0.31
10	0.05	180	0.61	0.39	2.21	0.28
10	0.1	200	1.08	0.82	2.76	0.43
10	0.25	140	1.27	1.25	2.69	0.35
0.05	10	60	0.32	0.70	2.73	0.41
0.1	10	140	0.66	0.63	2.55	0.32
0.25	10	140	0.54	0.94	3.88	0.42
10	10	100	1.23	1.21	3.00	0.48
0.05	-				0	0
0.1	-				approx 0.18	0.095
0.25	-				approx 0.25	0.11
10	-				approx 0.65	0.23

It is noteworthy that the decrease in bile flow is maximally induced even in the presence of 0.05nM vasopressin when 0.25nM glucagon is present. Thus this event appears to display a greater sensitivity to stimulation by hormones than the increase in bile flow rate.

The effect of low concentrations of vasopressin in combination with 10nM glucagon on bile flow.

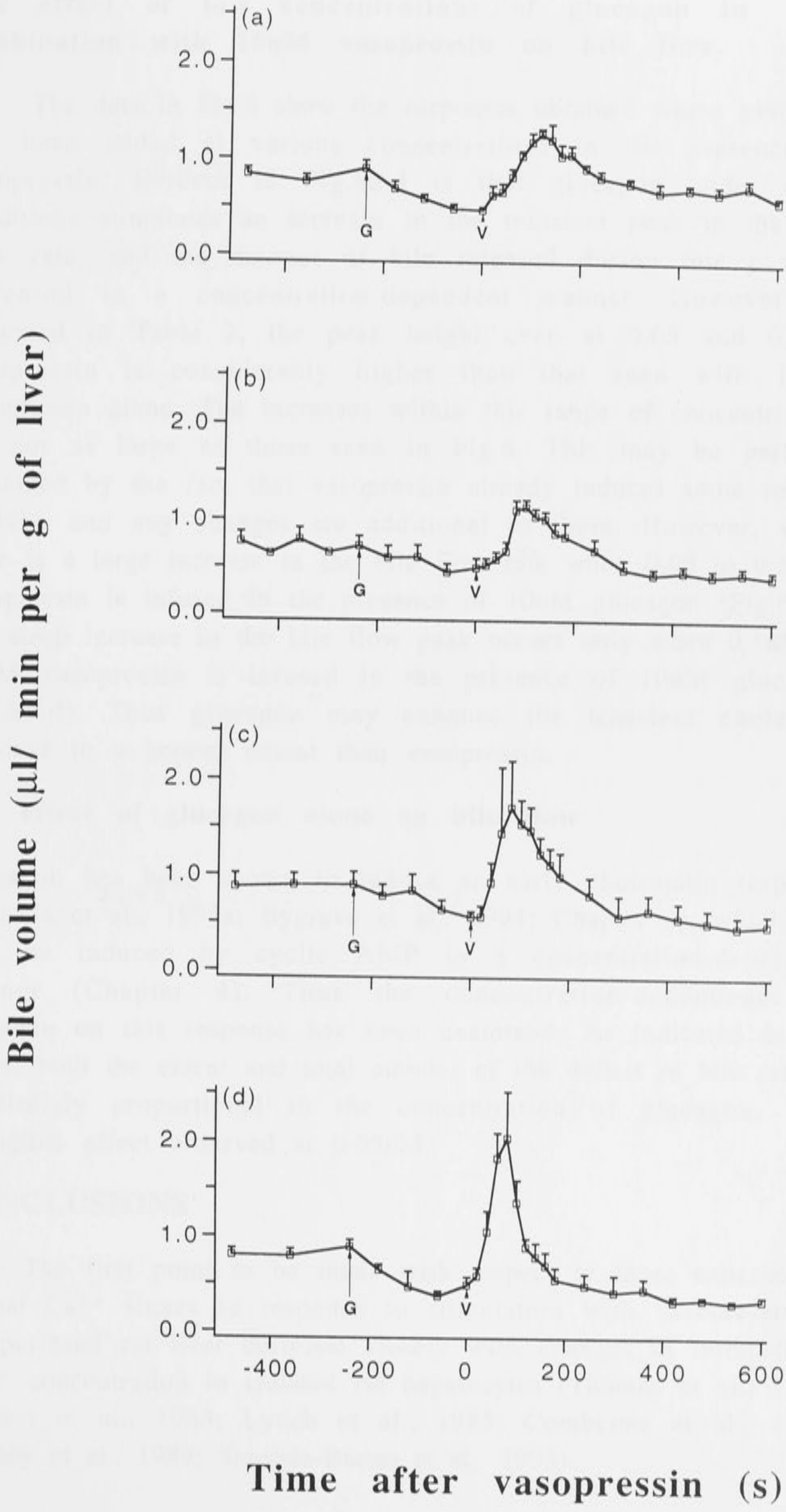
Evident in the data shown in Fig.7 is that in the presence of maximal concentrations of glucagon (10nM) (see e.g. Altin and Bygrave, 1986), only a small amount of vasopressin is required to induce a significant bile flow response. Even at the low concentrations of vasopressin (Fig.7a,b) there is a sizeable stimulation of bile flow under these conditions, which is equivalent to that seen with vasopressin concentrations of 0.25nM and above. The largest changes in the peak height occur as the concentration of vasopressin is increased from 0.05nM to 0.25nM. Both the increases in peak height and the associated increases in the amount of bile released have a similar relationship to changes in the concentration of vasopressin as has Ca^{2+} influx. As indicated in Fig.3a-d, the influx of Ca^{2+} is relatively stable at lower concentrations of the hormones, and the greatest change occurs between 0.05nM and 0.25nM vasopressin. The rate of change in the efflux rate (which is a more useful parameter to employ in this context than the maximum efflux rate, due to the efflux being terminated while still increasing by the influx in the presence of a high glucagon concentration), displays a similar dependence on vasopressin concentration. Thus it appears that Ca^{2+} mobilization, regardless of direction, is to a large extent determining the extent of bile release.

It is noteworthy that there are only minor changes in the subsequent decrease in bile flow rate across the whole range of vasopressin concentrations employed in this study. Thus this parameter appears to be very sensitive to the hormone treatments, the maximal response being observed with 0.025nM vasopressin under these conditions.

Thus the maximal glucagon concentrations synergistically enhance the peak in bile flow rate in response to vasopressin.

Fig.7. Changes in the bile flow rate in response to infusing with 10nM glucagon and varying low concentrations of vasopressin.

The changes in bile flow rate were measured concomitantly with the changes in Ca^{2+} concentration shown in Figure 3, in response to the infusion of 10nM glucagon with the following concentrations of vasopressin: (a) 0.025nM, (b) 0.05nM, (c) 0.1nM and (d) 0.25nM. Means ± S.E.M. from four to seven experiments are shown.


(a)
(b)
(c)
(d)
2.0
1.0
0.0
G
V
-400
-200
0
200
400
600
Bile volume (μl/ min per g of liver)
Time after vasopressin (s)

The effect of low concentrations of glucagon in combination with 10nM vasopressin on bile flow.

The data in Fig.8 show the responses obtained where glucagon has been added at various concentrations in the presence of vasopressin. Evident in Fig.8a-d is that glucagon under these conditions stimulates an increase in the transient peak in the bile flow rate, and the amount of bile released during this peak is increased in a concentration-dependent manner. However, as indicated in Table 2, the peak height even at 0.05 and 0.1nM vasopressin is considerably higher than that seen with 10nM vasopressin alone. The increases within this range of concentrations are not as large as those seen in Fig.6. This may be partially explained by the fact that vasopressin already induces some release of bile, and any changes are additional to these. However, while there is a large increase in the bile flow rate when 0.05 to 0.25nM vasopressin is infused in the presence of 10nM glucagon (Fig.6b,c), this steep increase in the bile flow peak occurs only when 0.1nM to 10nM vasopressin is infused in the presence of 10nM glucagon (Fig.8a-d). Thus glucagon may enhance the transient choleretic response to a greater extent than vasopressin.

The effect of glucagon alone on bile flow

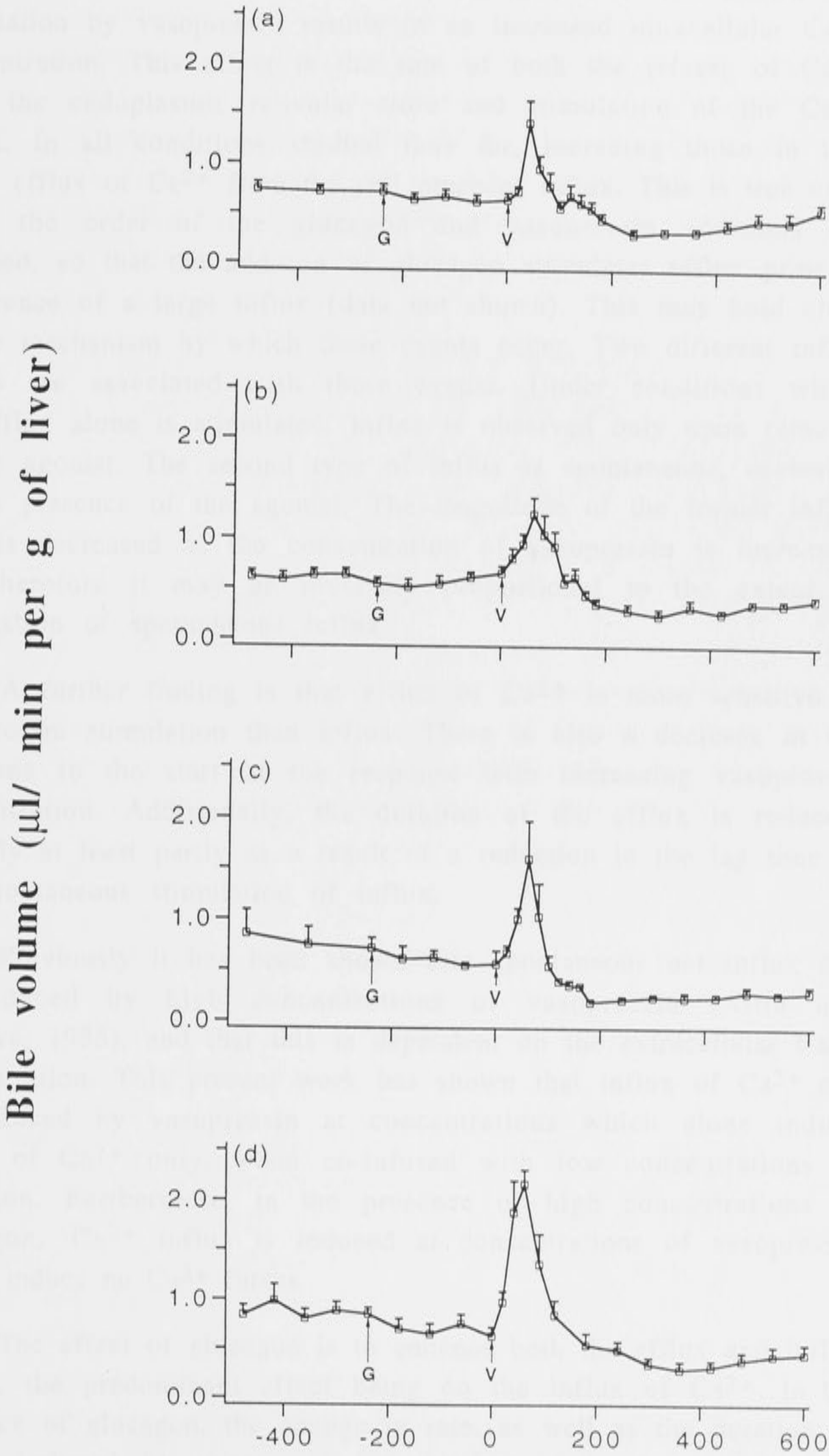
Glucagon has been shown to induce an early cholestatic response (Hamada et al., 1992a; Bygrave et al., 1994; Chapter 4), which can also be induced by cyclic AMP in a concentration-dependent manner (Chapter 4). Thus the concentration-dependence of glucagon on this response has been examined. As indicated in the Table, both the extent and total amount of the deficit in bile release is directly proportional to the concentration of glucagon, with negligible effect observed at 0.05nM.

CONCLUSIONS

The first point to be made with respect to these experiments is that Ca^{2+} fluxes in response to stimulation with vasopressin in the perfused rat liver correlate closely with changes in intracellular Ca^{2+} concentration in isolated rat hepatocytes (Thomas et al., 1984; Charest et al., 1985; Lynch et al., 1985; Combettes et al., 1986, Rooney et al., 1989; Sanchez-Bueno et al., 1993).

Fig.8. Changes in the bile flow rate in response to infusing with varying low concentrations of glucagon and 10nM vasopressin.

The changes in bile flow rate were measured concomitantly with the changes in Ca^{2+} concentration shown in Figure 4, in response to the infusion of glucagon at the following concentrations: (a) 0.05nM, (b) 0.1nM, (c) 0.25nM and (d) 10.0nM. Means ± S.E.M. from three to nine experiments are shown.


(a)
(b)
(c)
(d)
2.0
1.0
0.0
G
V
-400
-200
0
200
400
600
Bile volume (μl/ min per g of liver)
Time after vasopressin (s)

As indicated in the Introductory Review, it is well known that stimulation by vasopressin results in an increased intracellular Ca^{2+} concentration. This effect is the sum of both the release of Ca^{2+} from the endoplasmic reticular store and stimulation of the Ca^{2+} influx. In all conditions studied thus far, including those in this work, efflux of Ca^{2+} from the cell precedes influx. This is true even when the order of the glucagon and vasopressin additions are reversed, so that the addition of glucagon stimulates efflux prior to occurrence of a large influx (data not shown). This may hold clues to the mechanism by which these events occur. Two different influx events are associated with these events. Under conditions where net efflux alone is stimulated, influx is observed only upon removal of the agonist. The second type of influx is spontaneous, occurring in the presence of the agonist. The magnitude of the former influx type is decreased as the concentration of vasopressin is increased, and therefore it may be inversely proportional to the extent of stimulation of spontaneous influx.

A further finding is that efflux of Ca^{2+} is more sensitive to vasopressin stimulation than influx. There is also a decrease in the lag time to the start of the response with increasing vasopressin concentration. Additionally, the duration of the efflux is reduced, possibly at least partly as a result of a reduction in the lag time to the spontaneous stimulation of influx.

Previously it has been shown that spontaneous net influx can be induced by high concentrations of vasopressin (Altin and Bygrave, 1985), and that this is dependent on the extracellular Ca^{2+} concentration. This present work has shown that influx of Ca^{2+} can be induced by vasopressin at concentrations which alone induce efflux of Ca^{2+} only, when co-infused with low concentrations of glucagon. Furthermore, in the presence of high concentrations of glucagon, Ca^{2+} influx is induced at concentrations of vasopressin which induce no Ca^{2+} fluxes.

The effect of glucagon is to enhance both the efflux and influx events, the predominant effect being on the influx of Ca^{2+}. In the presence of glucagon, the change in rate, as well as the duration of influx induced by vasopressin, are enhanced in a concentration-dependent manner.

Some of the responses to Ca^{2+} fluxes observed with these hormones correlate with bile flow events. There is a vasopressin concentration-dependent increase in the height of the transient peak in bile flow, which always precedes a more sustained decrease in bile flow rate below basal levels, which has a similar dependence on vasopressin concentration. The reduction in the lag time to the onset of the decrease in bile flow may explain the decreased duration of the transient peak in bile flow with increasing concentrations of vasopressin.

In the presence of glucagon, the bile flow peak in response to vasopressin generally appears to be enhanced relative to the response in the absence of glucagon. When vasopressin is introduced in the presence of high concentrations of glucagon, the increase in bile flow follows a similar pattern to that in the absence of glucagon; only the extent of the responses is greater, the bile flow peak being induced even in the presence of very low (0.025nM) concentrations of vasopressin. Noteworthy is the similarity of the pattern of the bile flow changes with the pattern of Ca^{2+} influx under these conditions, with no further increase in the response being observed in either parameter when the vasopressin concentration is increased beyond 0.25nM in the presence of 10nM glucagon.

The decrease in bile flow rate is maximal at approximately 40% of the basal rate under all conditions of hormone stimulation. This maximal decrease inevitably follows a peak in bile flow rate which is greater than or equal to the maximal rate observed in the presence of vasopressin alone.

Thus in general the effect of vasopressin on bile flow correlates with Ca^{2+} fluxes induced by the hormone. This is most prominent in the presence of high concentrations of glucagon or in its absence, and implicates a role for Ca^{2+} mobilization in both the increase and decrease in bile flow observed under these conditions. However, a role for glucagon independent of its effect on Ca^{2+} mobilization in the enhancement of the transient increase in bile flow cannot be completely ruled out.

Chapter 4

Hormone-induced bile flow and hepato-biliary calcium fluxes are attenuated in the perfused liver of rats made cholestatic with ethynylestradiol *in vivo* and with phalloidin *in vitro*

Hormone-induced bile flow and hepato-biliary calcium fluxes are attenuated in the perfused liver of rats made cholestatic with ethynylestradiol *in vivo* and with phalloidin *in vitro*

INTRODUCTION

Vasopressin induces transient changes in bile flow, as well as mobilizing Ca^{2+}, and both these effects are potentiated by the action of glucagon (Hamada et al., 1992a; Bygrave et al., 1994). Thus cross-talk between these signalling pathways is implicated in the fine modulation of bile flow.

In the present chapter the extent to which the hormone-induced changes are affected in livers from rats made cholestatic by the administration of ethynylestradiol in vivo, or by the infusion of phalloidin in vitro was investigated. The effect of vasopressin and glucagon were examined under these conditions as well as the effect of these treatments on the cross-talk between these signalling systems.

EXPERIMENTAL

Outline of procedure

The general procedure adopted in these experiments was to perfuse livers from a group of rats in which cholestasis had been induced by daily injections of ethynylestradiol. The regime of hormone administration was as described in the general methods section. During the course of administering these hormone regimes, bile was collected to enable measurements of bile flow and calcium content in the samples taken. Measurements were also made of concomitant changes in the concentration of Ca^{2+}, oxygen and glucose in the outflow medium of the perfusate. A second group of rats was made cholestatic as above but after the fifth day, injections ceased and the animals were allowed to recover for 5 days from such treatment. The hormone regimes indicated above were then repeated on the perfused livers obtained from these 5-day 'post-treatment' animals.

Where phalloidin was infused into the perfused liver, the following protocol was followed, after having first determined that these conditions decreased basal bile flow by approximately 50% as seen in the experiments employing ethynylestradiol. Prior to infusing phalloidin, bile was collected over 5 minutes to determine a basal rate of flow. Control livers were treated in the same manner except that no phalloidin was infused. In experiments where phalloidin was used, bile was collected at 20 second intervals for 1 minute following vasopressin addition and then at 30 second intervals for a further minute.

Other measurements

Oxygen consumption and glucose release by the liver were determined as previously described (Hamada et al., 1992a). Total atomic calcium in the bile samples was measured using a Varian AA20 atomic absorption spectrophotometer. Samples were extracted with 1M perchloric acid (final concentration) and following centrifugation in an Eppendorf microfuge, the resulting supernatant was analysed for calcium in the presence of either $SrCl_2$ or $LaCl_3$ (0.2% final concentration) and KCl (0.1% final concentration). Bile flow was measured by weighing the bile fluid collected for each one minute; it was determined that 1µl is equivalent to 1mg wet weight. Calculations carried out to determine the relative lag time in each of the perfusate and bile flow cannulae, showed that these generally were within 5 to 10s of each other but in the cholestatic condition bile flow could lag by up to about 40s.

Expression of data

Rates of O_2 and Ca^{2+} concentration changes are expressed as µmol/min per g of liver. All experiments were performed at least three times. Where indicated, data are expressed as means $\pm$ S.E.M. for the number of independent experiments described. The significance of any hormone-induced effects within experimental groups was analysed against the pre-infusion (basal) data using the two-tailed t test for paired samples. Where the significance of differences in responses between different experimental groups was determined, the two-tailed t-test of Welch (Afifi and Azen,

1979) for unpaired samples was used. This test does not assume equality of the variances in the two groups.

It should be noted that although data for bile flow, bile calcium and perfusate glucose output have been expressed on a liver weight basis, we observed that the liver weight to body weight ratio generally increased by a factor of approximately 20% following cholestasis. This could to some extent be accounted for by the decrease in body weight observed in this study. In a carefully controlled study by Hornstein et al (1992), it was shown that ethynylestradiol-induced liver enlargement was due to both hypertrophy as well as hyperplasia. It has also been shown that phalloidin can cause liver enlargement (for a discussion see e.g. Frimmer, 1987).

RESULTS

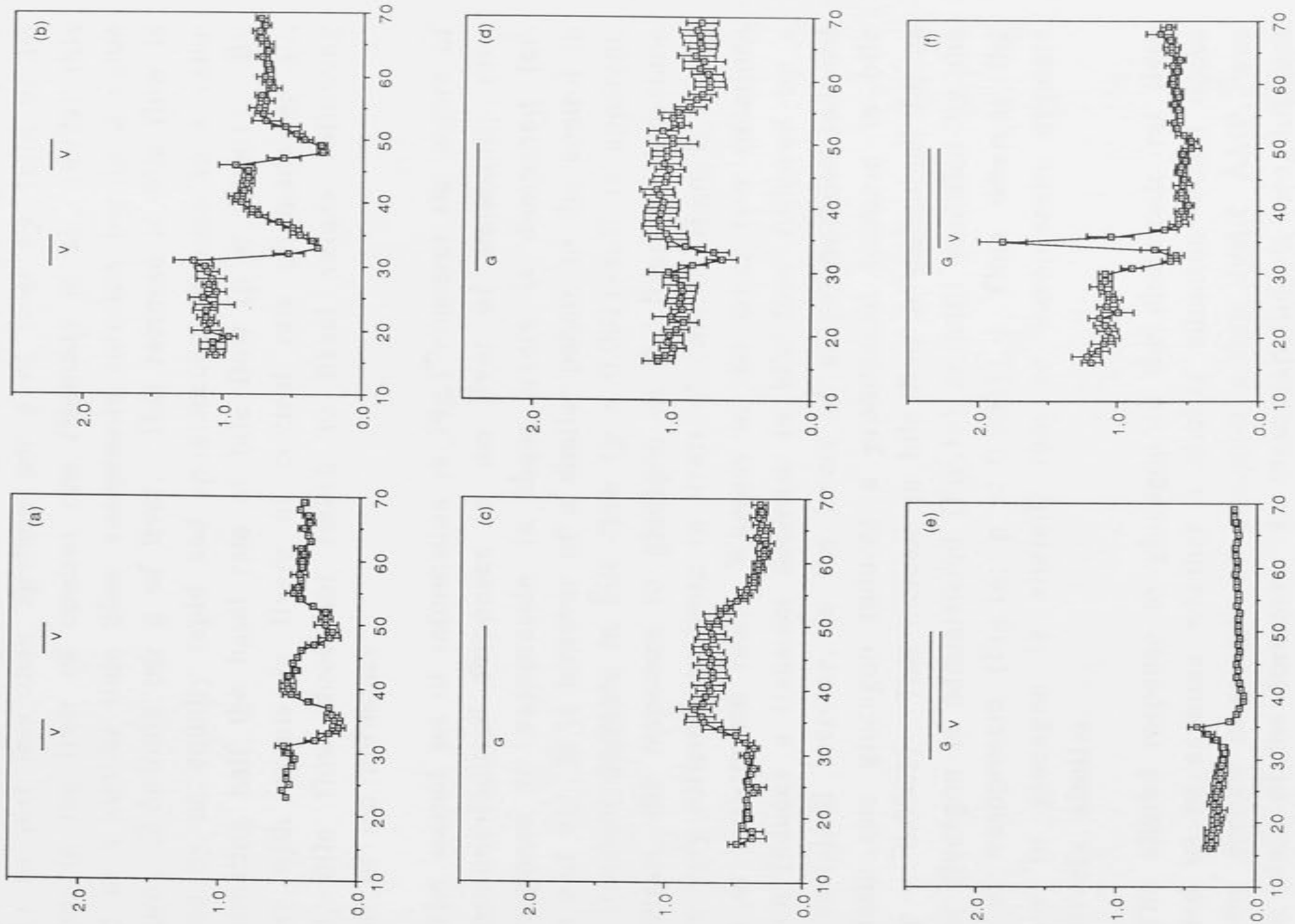
Of general note is the observation that the basal bile flow rate in the perfused livers of treated rats is of the order of 0.5µl/min per g of liver or at least half the basal rate seen in livers of rats 5 days post-treatment (see Fig.1). This confirms that the injection regime did induce cholestasis. These latter rates of bile flow are similar to those observed in the livers of control rats (ie. those not subjected to any treatment with the cholestatic agent, see eg. Hamada et al., 1992a).

Effects of vasopressin and glucagon on bile flow in the perfused liver of cholestatic and post-treated rats.

The first set of information revealed in Fig.1 is that the responses in bile flow to each of the three regimes of hormone administration in the livers of post-treated rats (right column of figure set), are similar to what is observed in the liver of control rats (cf. Hamada et al., 1992a). Particularly evident is the sharp diminution in bile flow ($p < 0.0001$) following the administration of each of the two pulses of vasopressin. As in the liver of control rats (cf. Hamada et al., 1992a), this returns to basal levels after approx. 10 min irrespective of the length of time for which the hormone is administered (data not shown). Infusion of glucagon (Fig.1d) transiently attenuates bile flow ($p = 0.002$) reaching its nadir at approx. 2 min; by 5 min, bile flow has reverted to a rate that is slightly but significantly greater ($p < 0.0001$) than basal.

Fig.1. Effects of vasopressin and glucagon, administered separately or together, on bile flow in the perfused liver of ethynylestradiol-treated rats and of post-treated rats.

One group of rats was made cholestatic by the daily injection for 5 days of ethynylestradiol and another group allowed to recover for 5 days following such injections. Livers from both groups were perfused with Krebs-Henseleit bicarbonate medium containing 1.3mM Ca^{2+}. After a pre-perfusion period of 20min, two pulses of 10nM vasopressin (a,b), or a single pulse of 10nM glucagon (c,d) or a combination of both hormones (e,f) were infused for the times indicated by the horizontal bars. Bile flow was measured as described in the experimental section. Data (means $\pm$ S.E.M. of at least four independent experiments) for cholestatic rats is shown in (a), (c) and (e) and for post-treated rats in (b), (d) and (f). For further details see the Experimental section.



Perfusion time (min)

Bile volume (μl/min per g of liver)

The most obvious changes in bile flow post-treatment occur following the co-administration of glucagon and vasopressin (Fig.1f). Immediately following the administration of vasopressin to livers where glucagon already is being administered, there rapidly occurs a striking increase in bile flow that peaks ($p < 0.0001$) to just less than 2µl/min per g of liver by 1min of its infusion; in the liver of control rats (Hamada et al., 1992a) this peaked at a similar time after vasopressin infusion but to a value of approx. 2.5µl/min per g of liver. This increase in bile flow is followed by an equally rapid and spontaneous decrease to a value approximating half the basal rate of bile flow ($p < 0.0001$). By contrast with events in livers of control rats (Hamada et al., 1992a), bile flow does not return to basal values following removal of the hormones.

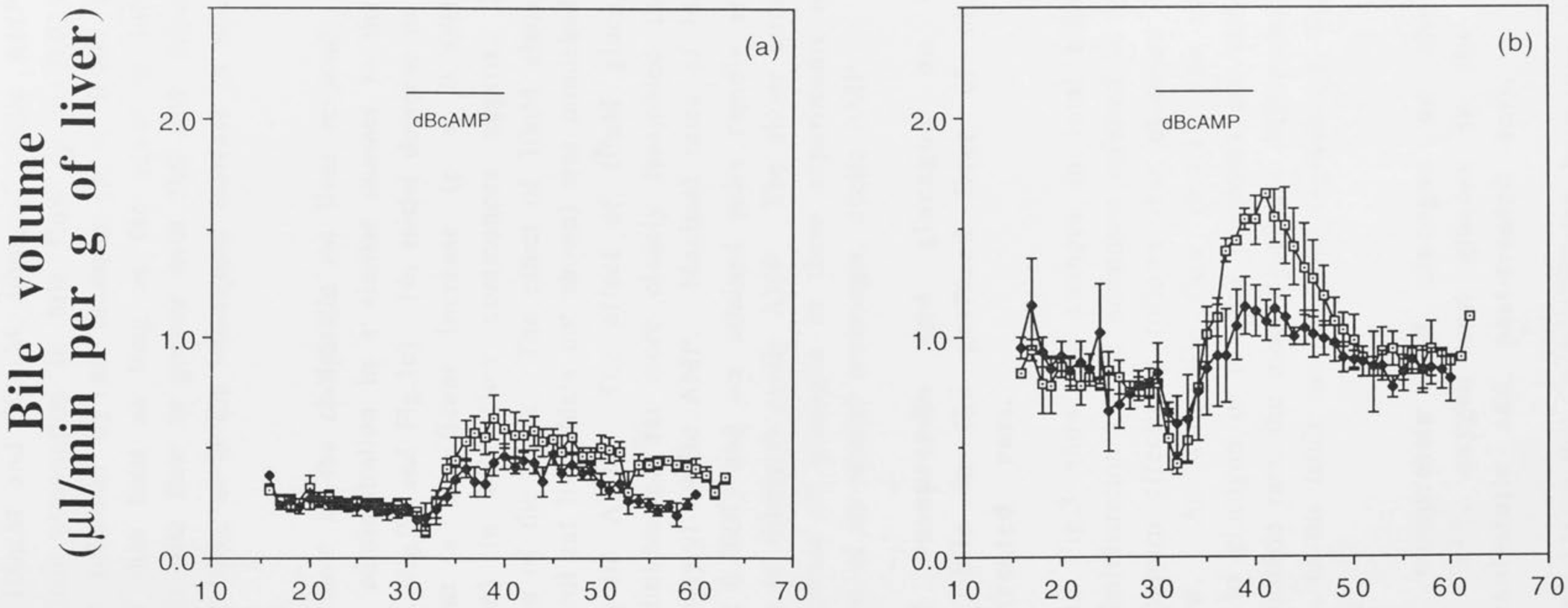
The second set of information in Fig.1 concerns the nature of the hormone-induced responses in the liver of cholestatic rats. The response to vasopressin in these livers is dampened (cf. Figs.1a and b). It is however of a similar pattern to the control in that a transient decrease in bile flow ($p < 0.001$) still is apparent. By contrast, the responses to glucagon in the liver of cholestatic rats are very different to those in livers of control (Hamada et al., 1992a) or post-treated rats. Whereas in the latter two situations glucagon induces a transient decrease in bile flow followed by a slight sustained increase, in the livers of ethynylestradiol-induced cholestatic rats glucagon induces a pronounced increase in bile flow ($p < 0.0001$). This increase in bile flow is maintained for as long as glucagon is administered (Fig.1c) or until modified by the action of vasopressin (Fig.1e; $p < 0.0001$). Thus whereas the response to glucagon is altered, that to vasopressin appears qualitatively similar.

The altered response to glucagon in the cholestatic rat liver prompted us to examine whether a similar situation would occur when the second messenger of glucagon action cyclic AMP, was used in place of the hormone. In these experiments two different concentrations of the permeable analogue dibutyryl cyclic AMP were employed.

Data in Fig.2b, obtained from control rats, show that dibutyryl cyclic AMP induces a dose-dependent response in bile

Fig.2. Effect of dibutyryl cyclic AMP on bile flow in the perfused liver of ethynylestradiol-treated rats and of post-treated rats.

The experimental procedure was as described for Fig.1 except that dibutyryl cyclic AMP (dBcAMP) was administered at 100μM (closed symbols) or 500μM (open symbols) as indicated by the horizontal bars. Data (means ± S.E.M. of at least four independent experiments) for cholestatic rats is shown in (a) and for post-treated rats in (b). For further details see the Experimental section.


Bile volume
(μl/min per g of liver)
(a)
dBcAMP
2.0
1.0
0.0
10
20
30
40
50
60
70
(b)
dBcAMP
2.0
1.0
0.0
10
20
30
40
50
60
70
Perfusion time (min)

flow; both 100μM and 500μM dibutyryl cyclic AMP induce an initial transient attenuation in bile flow ($p = 0.035$ and 0.01, respectively) followed by an increase ($p < 0.001$ and 0.0001, respectively) that lasts as long as the agent is infused. The attenuation in bile flow is greater with 500 μM dibutyryl cyclic AMP ($p = 0.076$), as is the subsequent increase in bile flow ($p < 0.001$).

Bile flow in the cholestatic rat liver responds to dibutyryl cyclic AMP administration in a similar manner to that seen with glucagon (cf. Fig.2a and Fig.1c). No initial decrease in bile flow is observed but a significant increase ($p = 0.0001$ for each concentration) in bile flow commences approx. 2min. after administration of the agent. The effect of 10nM glucagon on bile flow in control rat liver (data not shown) was mimicked by 100μM dibutyryl cyclic AMP. The effect of 10nM glucagon in the ethynylestradiol-treated rat more closely resembled the effect of 500 μM dibutyryl cyclic AMP. Maximal rates of bile flow are greater ($p < 0.0001$) and are attained more rapidly at the higher concentration of dibutyryl cyclic AMP. The above strongly implies that the response to glucagon in these experiments occurred via the generation of its second messenger, cyclic AMP.

Effects of vasopressin and glucagon on calcium content of bile in the perfused liver of cholestatic and post-treated rats.

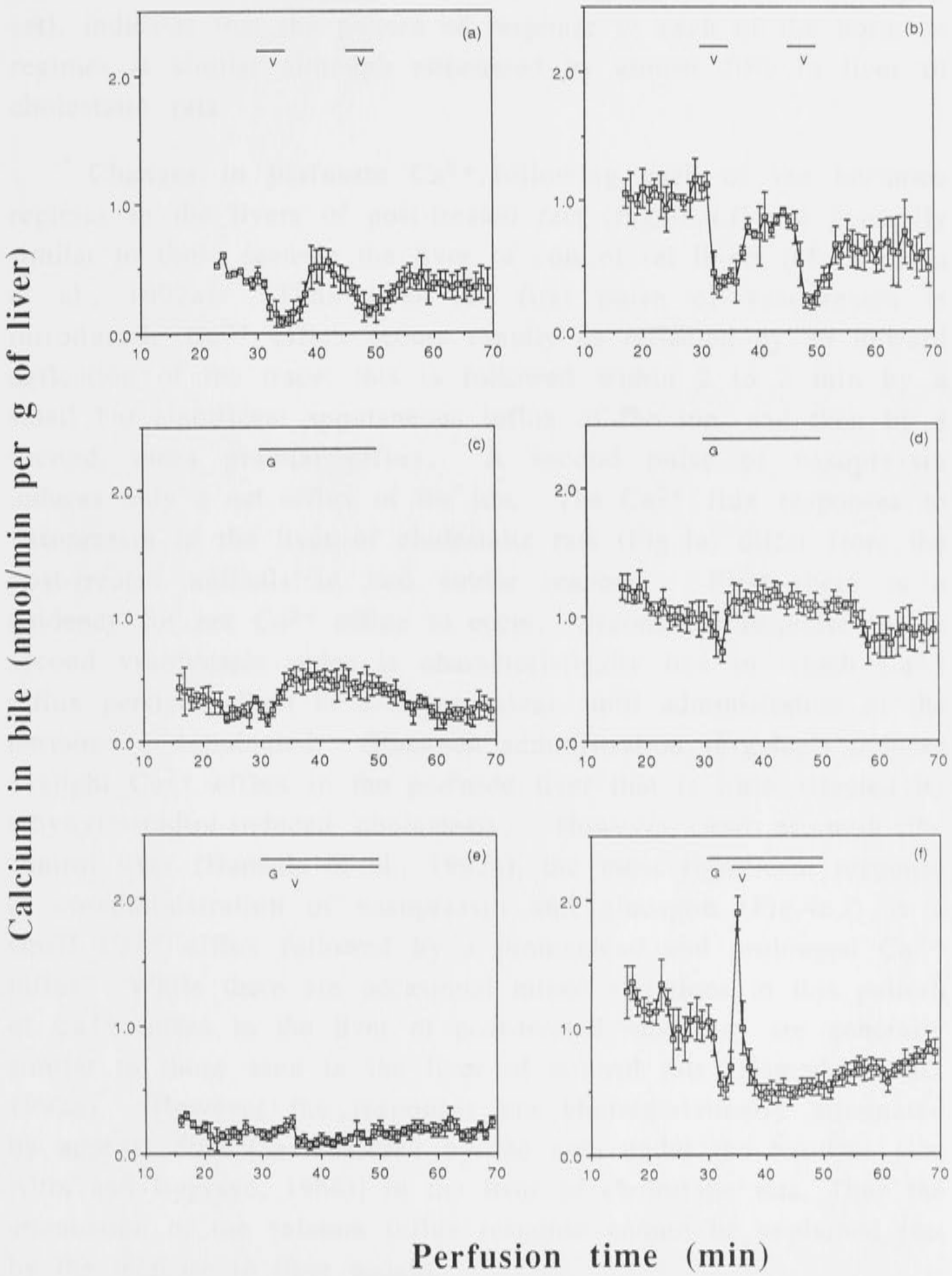
Data in Fig.3 show the changes in total biliary calcium following administration of the hormones referred to above. The underlying pattern effectively follows that observed for changes in bile flow. As with bile flow (Fig.1), the responses to vasopressin, to glucagon or to both hormones are different in the liver of cholestatic rats; the most significant differences are seen in the responses to the latter two hormone regimes (cf. Figs.3c and d, Figs.3e and f).

Effects of vasopressin and glucagon on changes in perfusate Ca^{2+}, oxygen and glucose in the perfused liver of cholestatic and post-treated rats.

It is well known that Ca^{2+}-mobilising hormones and glucagon stimulate glycogenolysis, oxygen uptake and Ca^{2+} fluxes in rat

Fig.3. Effects of vasopressin and glucagon, administered separately or together, on the concentration of calcium in the bile in the perfused liver of ethynylestradiol-treated rats and of post-treated rats.

The concentration of calcium in the bile was measured as described in the experimental section using the perfused livers shown in Fig.1. Data (means $\pm$ S.E.M. of at least four independent experiments) for cholestatic rats is shown in (a), (c) and (e) and for post-treated rats in (b), (d) and (f). For further details see the Experimental section.


(a)
V
V
(b)
V
V
(c)
G
(d)
G
(e)
G
V
(f)
G
V
2.0
1.0
0.0
10
20
30
40
50
60
70
Calcium in bile (nmol/min per g of liver)
Perfusion time (min)

liver. The extent to which these responses might be altered in rats treated with ethynylestradiol were therefore investigated. Comparison of the oxygen traces obtained from livers of cholestatic rats in Fig.4 (left column of figure set) with those obtained from livers of post-treated rats (right column of figure set), indicates that the pattern of response to each of the hormone regimes is similar although attenuated by almost 50% in liver of cholestatic rats.

Changes in perfusate Ca^{2+}, following each of the hormone regimes in the livers of post-treated rats (Fig.4b,d,f) are generally similar to those seen in the liver of control rat livers (cf. Hamada et al., 1992a). Thus after the first pulse of vasopressin is introduced, Ca^{2+} efflux occurs rapidly as reflected by an upward deflection of the trace; this is followed within 2 to 3 min by a small but significant spontaneous influx of the ion, and then by a second, more gradual efflux. A second pulse of vasopressin induces only a net efflux of the ion. The Ca^{2+} flux responses to vasopressin in the liver of cholestatic rats (Fig.4a) differ from the post-treated animals in two subtle respects. First there is a tendency for net Ca^{2+} efflux to occur. Second the response to the second vasopressin pulse is characteristically one in which Ca^{2+} efflux persists, albeit to a lesser extent, until administration of the hormone is terminated. Glucagon administration (Fig.4c,d) induces a slight Ca^{2+} efflux in the perfused liver that is little affected by ethynylestradiol-induced cholestasis. However, and as with the control liver (Hamada et al., 1992a), the most significant response to co-administration of vasopressin and glucagon (Fig.4e,f) is a small Ca^{2+} efflux followed by a pronounced and prolonged Ca^{2+} influx. While there are occasional minor variations to this pattern of Ca^{2+} influx in the liver of post-treated rats, they are generally similar to those seen in the liver of control rats (Hamada et al., 1992a). However the responses are characteristically attenuated by approx. 50% [as measured by the area under the baseline (see Altin and Bygrave, 1986)] in the liver of cholestatic rats. Thus the attenuation of the calcium influx response cannot be explained just by the increase in liver weight.

Glucose release in response to the hormone regimes adopted in this work in livers of cholestatic and post-treated rats is shown in Table 1. Basal glucose output (ie. that seen prior to hormone

Fig.4. Effects of vasopressin and glucagon, administered separately or together, on the change in perfusate Ca^{2+} and oxygen consumption in the perfused liver of ethynylestradiol-treated rats and of post-treated rats.

The concentrations of Ca^{2+} (continuous trace) and of oxygen (dotted trace) in the perfusate were measured as described in the experimental section using the perfused livers shown in Fig.1. Data (typical traces obtained for perfusate Ca^{2+} and for oxygen) for cholestatic rats is shown in (a), (c) and (e) and for post-treated rats in (b), (d) and (f). Note the change in scale for (e) and (f). For further details see the Experimental section. Basal rates of oxygen uptake in livers of both cholestatic and post-treated rats were each approx. 1.6µmol/min per g of liver.

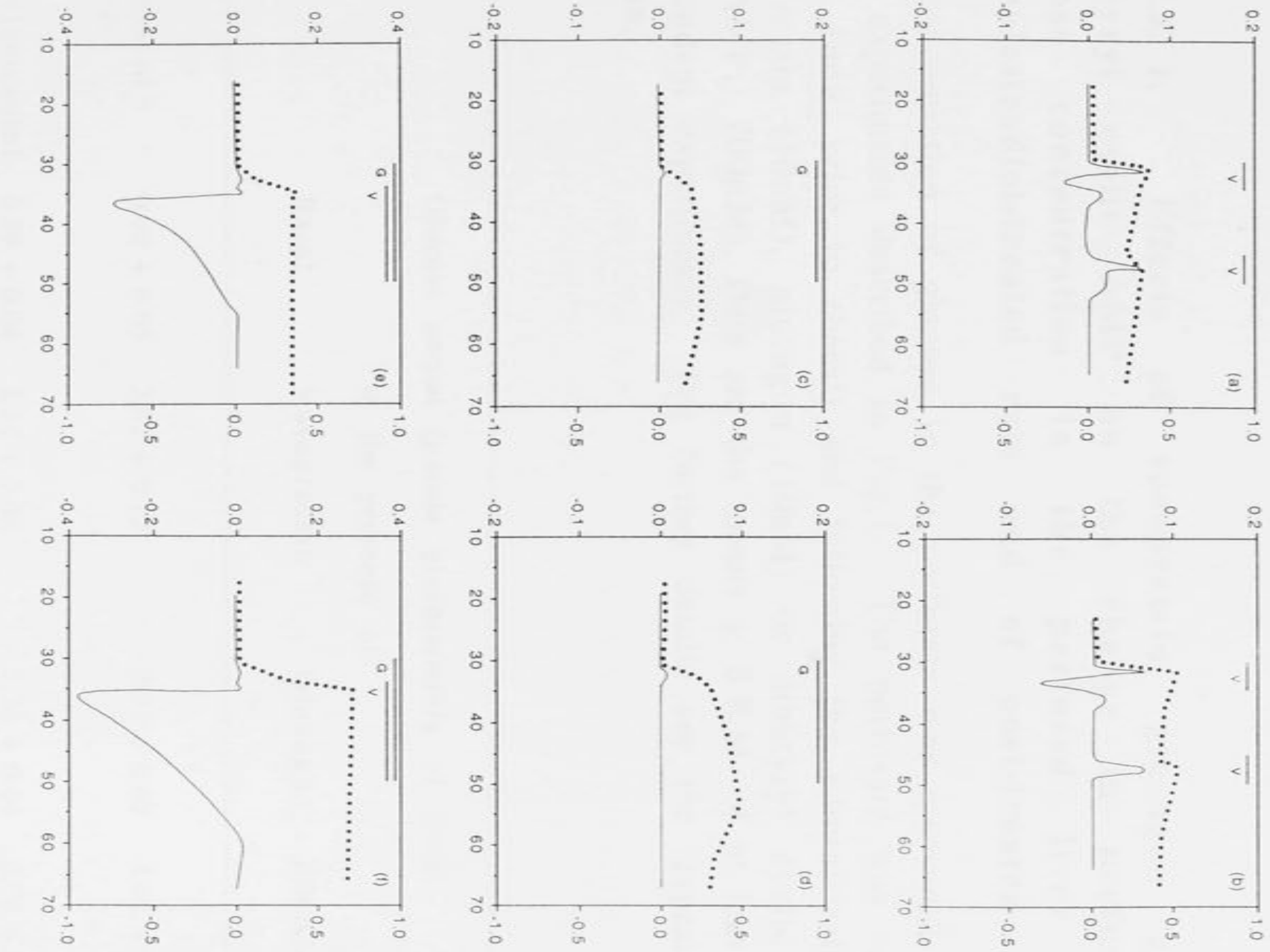

Change in perfusate [Ca2+] (μmol/min per g of liver)
Change in perfusate [O2] (μmol/min per g of liver)
Perfusion time (min)
(a)
(b)
(c)
(d)
(e)
(f)
V
G

TABLE 1. Effects of vasopressin, glucagon and dibutyryl cyclic AMP on the change in perfusate glucose concentration in the perfused liver of ethynylestradiol-treated rats and of post-treated rats.

The concentration of glucose in the perfusate was measured in the same experiments described in Fig.1. The perfusate was sampled every 1min. prior to (basal) and following the administration of vasopressin (10nM), glucagon (10nM) or dibutyryl cyclic AMP (dBcAMP; 100μM). Data are the means ± S.E.M. of at least three independent experiments. For further details see the Experimental section.

	Glucose output (μmole glucose/min/g of liver) in the presence of:			
	Basal	Vasopressin	Glucagon	dBcAMP
Post-treated rat liver	0.98 + 0.05	2.62 + 0.12	3.41 + 0.07	3.62 + 0.24
Ethynylestradiol-treated rat liver	0.89 + 0.04	2.34 + 0.08	2.76 + 0.04	2.78 + 0.08

administration), is similar in all situations, including that in livers of control rats (Hamada et al., 1992a) and is approx. 10-20% less in ethynylestradiol-treated animals. Hormone-induced glucose output is attenuated slightly in the liver of cholestatic rats. Basal hepatic oxygen uptake is similarly unaffected in the cholestatic rat but the hormone-induced oxygen uptake is attenuated (Fig.4).

Effects of phalloidin

The acute administration of phalloidin to the perfused rat liver had no significant effect on perfusate Ca^{2+} fluxes, glucose output or bile calcium (data not shown). The effects of its acute administration to the perfused rat liver on basal and hormone-induced bile flow are shown in Fig.5b, c and d. A rapid decline in bile flow occurs soon after phalloidin is administered. This reaches about 50% of the initial basal value by the time phalloidin is removed. By contrast with the control (Fig.5a), subsequent administration of glucagon does not decrease bile flow (Fig.5b,d). Vasopressin, on the other hand, induces a significant increase ($p = 0.001$) in bile flow (Fig.5c). The magnitude of this increase is similar to that observed following the prior administration of glucagon (Fig.5b; $p = 0.004$). In both situations, this increase is transient and followed by a rapid attenuation ($p < 0.0001$) of bile flow as observed with the control (Fig.5a).

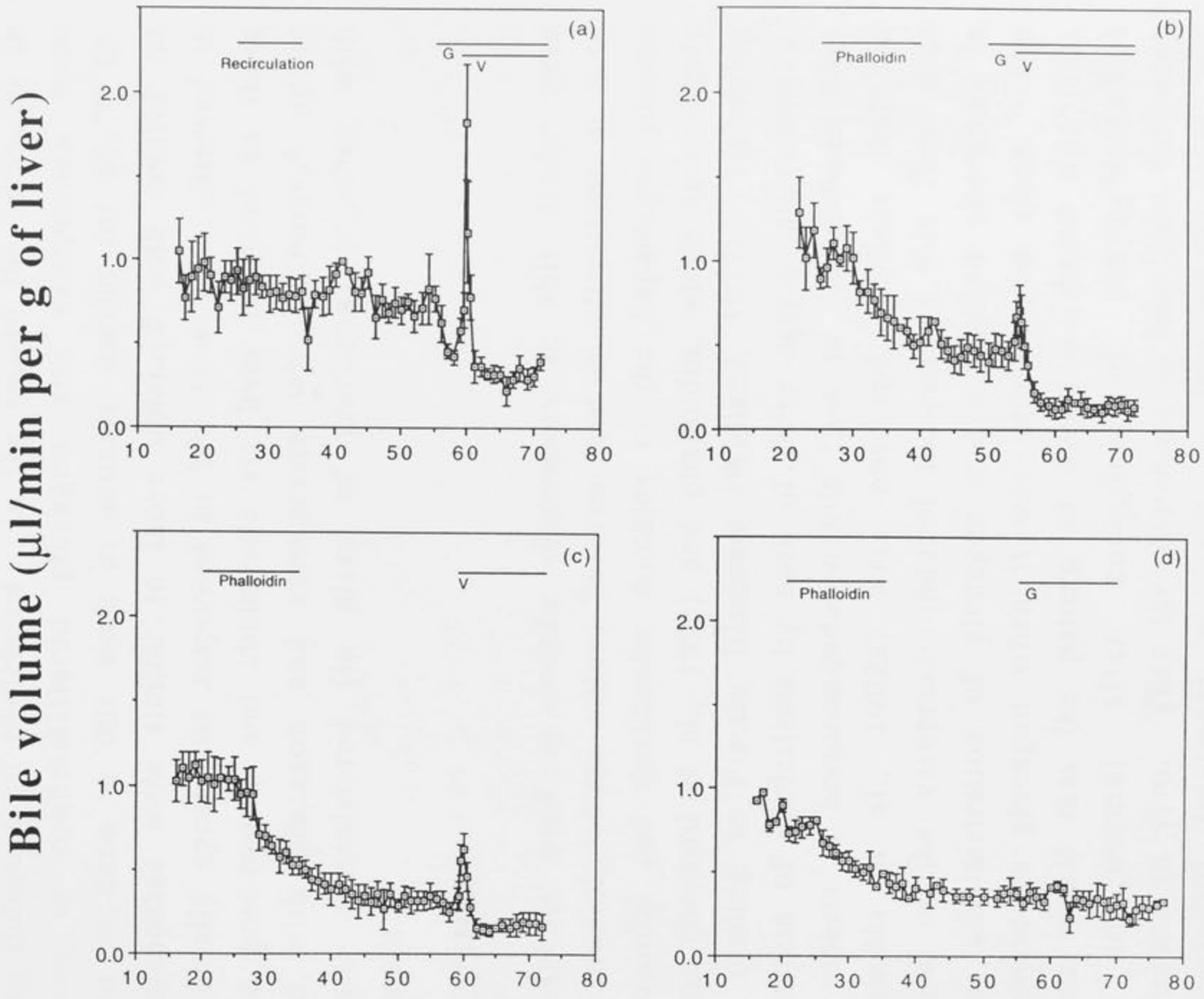
In other experiments (data not shown) rats were treated with phalloidin *in vivo* over 5 days. An attenuated response to the administration of glucagon and vasopressin was observed in the livers of these rats similar to that seen in Fig.5b. This similarity in action of phalloidin on bile flow when administered acutely or to the intact animal, contrasts with that of ethynylestradiol. When administered acutely, ethynylestradiol had no effect on either basal bile flow or on that induced by the synergistic action of glucagon and vasopressin (data not shown).

Other experiments data of which are not shown

An assessment was made of the extent to which the daily administration of ethynylestradiol affected feed consumption and body weight of the rats. Over the 5 days, food consumption was observed to decrease by up to 50% and body weight by some 15%. The possibility arose that the decrease in food consumption could

Fig.5. Effects of acute infusion of phalloidin on vasopressin and glucagon-induced bile flow in the perfused liver of control rats.

Livers from control rats were perfused with Krebs-Henseleit bicarbonate medium containing 1.3mM Ca^{2+}. Phalloidin was present in the perfusate at a concentration of 1.37mg/litre (ie. 1.7μM) for 15 minutes; it was infused for 5 minutes, and recirculated for 10 minutes after which the system was reverted to a non-recirculating mode. Bile flow was measured as described in the Experimental section. The concentrations of glucagon and vasopressin were as for Fig.1). For further details see the Experimental section.


(a)
Recirculation
G
V
(b)
Phalloidin
G
V
(c)
Phalloidin
V
(d)
Phalloidin
G
2.0
1.0
0.0
10
20
30
40
50
60
70
80
Bile volume (μl/min per g of liver)
Perfusion time (min)

lead to a physiological situation akin to starvation and that this might contribute to the results observed in the liver of cholestatic animals. Two sets of experiments were undertaken to test this. In the first the glycogen content (mg/g of liver, n=4) of the liver was measured prior to and following the 5-day treatment; these values were 9.51 $\pm$ 0.59 for 5-day ethynylestradiol-treated rats and 8.87 $\pm$ 0.57 for propylene glycol (vehicle) or untreated rats. Thus if anything, there was a slight increase in liver glycogen content following induction of cholestasis. In the second the responses in bile flow to coadministered glucagon and vasopressin were measured as done in this work in animals starved for 65-70 hr. These responses were similar to those obtained with control rat liver. Finally synergistic responses in bile flow were observed in both the post-treated and cholestatic rat liver identical to those obtained with glucagon and vasopressin when dibutyryl cyclic AMP was administered (in place of glucagon) together with vasopressin.

CONCLUSIONS

Agents used to induce cholestasis in this study were ethynylestradiol, whose actions in vivo lead to alterations at both the sinusoidal and canalicular domains of the hepatocyte plasma membrane (Bossard et al., 1993) and phalloidin, which acts largely through binding to F-actin filaments (Wieland, 1977). Following the induction of cholestasis by each of these agents, alterations in several aspects of hormone-induced bile flow in the perfused liver (cf. Hamada et al., 1992a) were observed. These included attenuation of the vasopressin-induced increase in bile flow after the prior administration of glucagon and a marked alteration in the response to glucagon when it was administered alone. The data also reveal that the pattern of hormone-induced bile flow reverts to normal after cessation of ethynylestradiol administration in vivo. Thus the alterations to bile flow following ethynylestradiol treatment as used in this work were not permanent. Hormone-stimulated glucose output, oxygen uptake and Ca^{2+} fluxes were not greatly attenuated in either ethynylestradiol- or phalloidin-induced cholestasis. This suggests (i) that the events leading to cholestasis did not produce any substantial deleterious effects on hormone-induced changes in hepatocyte metabolism and (ii) that the ability of vasopressin to

generate its second messenger (inositol trisphosphate) and mobilise intracellular Ca^{2+} was not compromised.

It is well established that a major action of vasopressin in hepatocytes involves the mobilisation of both intracellular and extracellular Ca^{2+} and that this ion is crucial for basal bile secretion (1). The finding in this study in the cholestatic rat (a) that biliary calcium responses are similar to those of bile flow and (b) that Ca^{2+}-sensitive glucose output is not significantly altered following vasopressin action, would suggest that perturbations in vasopressin-induced calcium homeostasis are most likely not a primary cause of the pathophysiology of the cholestasis observed herein.

The early transient increases in bile flow induced by the synergistic action of glucagon plus vasopressin were affected differently in the two cholestasis-inducing strategies used in this work (data are summarised in Table 2). On the one hand, the ability of vasopressin to induce a transient increase in bile flow was totally obliterated in rats treated in vivo with ethynylestradiol. On the other, vasopressin still was able to transiently stimulate bile flow albeit to a lesser degree in rat liver treated acutely with phalloidin.

The effects of these agents on responses in bile flow induced by glucagon alone differed from those induced by vasopressin alone. A particularly significant finding is that glucagon appears to have two actions on bile flow that manifest themselves differently following ethynylestradiol and phalloidin treatment. In livers of control (Hamada et al., 1992a) and post-treated rats (Fig.1d), glucagon administration leads to an early transient attenuation of bile flow followed by a small but significant increase in bile flow. Following treatments with both ethynylestradiol (Fig.1c) and phalloidin (Fig.5d), glucagon failed to attenuate bile flow. Previously we showed (Hamada et al., 1992b) that T-CDCA also attenuates the glucagon-induced responses (see Table 2). Glucagon also failed to synergistically enhance vasopressin-induced increases in bile flow (Fig.1e, Fig.5b).

By contrast with this, administration of glucagon (Fig.1c) or of the permeable cyclic AMP analogue dibutyryl cyclic AMP

TABLE 2. Summary of effects of glucagon and vasopressin on early bile flow in perfused livers from normal rats and in those made cholestatic in vivo and in vitro
Unless indicated otherwise descriptions are from experiments performed in this paper.

Treatment	Response to:		
	Vasopressin	Glucagon	Vasopressin + Glucagon
None (control)+	Transient increase then transient decrease	Transient decrease then slight increase	Enhanced increase then sustained decrease maintained for period of hormone input
Estradiol administered in vivo	Transient increase lost	Transient decrease lost; large sustained increase	Transient increase lost; sustained decrease maintained beyond hormone output
Estradiol administered in vivo then recovered	Transient increase attenuated then transient decrease	Transient decrease then slight increase	Enhanced increase then sustained decrease maintained for period of hormone input
Phalloidin administered acutely	Transient responses similar to control	Transient decrease lost; no subsequent increase	Transient increase partially lost
TCDCA administered acutely*	Transient responses similar to control	Transient decrease attenuated	Transient increase partially lost

+ Control data from Hamada et al (1992a)

* These data from Hamada et al (1992b)

(Fig.2a) induced a significant increase in bile flow in ethynylestradiol-treated rats indicating that glucagon is acting via the generation of cyclic AMP. This response to glucagon was not seen following the acute administration of phalloidin (Fig.6d) or of T-CDCA (see Table 2). An increase in bile flow of similar magnitude is produced when a high concentration of dibutyryl cyclic AMP is infused, both in the ethynylestradiol-treated and control liver. The flow of bile increases approximately 2-fold with 500 μM dibutyryl cyclic AMP, as found also by Hamlin et al (1990). Phalloidin, which binds to F-actin (Wieland, 1977), attenuates the actions of both glucagon and vasopressin (Fig.5).

Thus when intrahepatic cholestasis is induced by ethinylestradiol, the transient increases in bile flow induced by vasopressin are abolished. By contrast, the decreases in bile flow induced by glucagon or cyclic AMP are abolished, while the increase in bile flow is substantially enhanced.

The data support an earlier view (Hamada et al., 1992a,b) that these transients may be relevant to the physiology of bile formation and to the pathophysiology of cholestasis.

Chapter 5

Restoration of hormone-induced bile flow and hepatobiliary calcium by acute administration of bile acids to the perfused liver of experimentally-induced cholestatic rats

Restoration of hormone-induced bile flow and hepatobiliary calcium by acute administration of bile acids to the perfused liver of experimentally-induced cholestatic rats

INTRODUCTION

As already mentioned in this thesis, an issue of current interest in the area of hepato-biliary physiology is the means by which bile flow is regulated. A number of hormones which rapidly mobilize hepatic Ca^{2+}, an ion crucial for the maintenance of basal bile flow (Owen, 1977; Reichen et al., 1985), can also modulate the flow of bile. The actions of Ca^{2+}-mobilising hormones in liver can be modified by glucagon. This occurs through 'cross-talk' between the second messengers generated by each of these hormones (reviewed in Bygrave and Benedetti, 1993). Earlier work has shown that such cross-talk modulates bile flow (Hamada et al., 1992a; Bygrave et al., 1994; Chapter 1). The pattern of this modulation is altered following the acute administration of cholestatic bile acids to perfused rat liver (Hamada et al., 1992b) as well as in rats made cholestatic by the administration of ethynylestradiol in vivo or phalloidin in vitro (Chapter 4). These observations support the notion that such short-term hormone-induced modulation of bile flow may be relevant to the physiology of bile flow and to the pathophysiology of cholestasis.

Bile acids are continuously flowing through the liver as part of the enterohepatic circulation, either as a result of their synthesis therein or in the course of their passage from the blood plasma to the bile (for a review see Erlinger, 1993 and the Introductory Review). Certain bile acids have been found to have an ameliorating effect in some cholestatic conditions *in vivo* (see e.g. Queneau and Montet, 1994). Moreover it is known that particular bile acids are able to mobilise Ca^{2+} in hepatocytes (Combettes et al., 1989, 1992; Beuers et al., 1993a,b; Bouscarel., 1993; Thibault and Ballet, 1993) and in perfused rat liver (Hamada et al., 1992b). Consequently an important issue that arises is the mechanistic link between hormone action, Ca^{2+} mobilisation and bile flow.

In light of these and the above-mentioned issues it was considered important to examine the influence of bile acids on hormone-modulated bile flow and biliary calcium in the livers of experimentally-induced cholestatic rats. Here it is shown that the acute administration of G-UDCA, T-UDCA and UDCA to livers of cholestatic rats can restore these modulating responses but with differing degrees of efficacy in the two models of cholestasis investigated.

EXPERIMENTAL

Outline of procedure

The general procedure adopted in these experiments was to perfuse livers from a group of rats in which cholestasis had been induced by daily injections of ethynylestradiol. The regime of hormone administration was as described in the General Methods section. During the course of administering these hormone regimes, bile was collected to enable measurements of bile flow and calcium content in the samples taken. Measurements were also made of concomitant changes in the concentration of Ca^{2+}, oxygen and glucose in the outflow medium of the perfusate. The data for the former two parameters are not shown.

Other measurements

Oxygen consumption, glucose release and total atomic calcium in the bile samples were determined as described in the experimental section of the previous chapter (see also Hamada et al.,1992a).

RESULTS

The data presented show the effects of infusing 100μM G-UDCA or 100μM T-UDCA to perfused livers of control rats (left-hand column) or those made cholestatic by the administration in vivo of ethynylestradiol (right-hand column). Preliminary experiments indicated that the conjugated bile acids were generally more effective than UDCA in promoting bile flow in the perfused liver of cholestatic rats. Although efforts were concentrated on a study of these compounds, some experiments with UDCA also are reported.

Effect of G-UDCA and T-UDCA on basal and hormone-induced bile flow in livers from control rats and those made cholestatic with ethynylestradiol

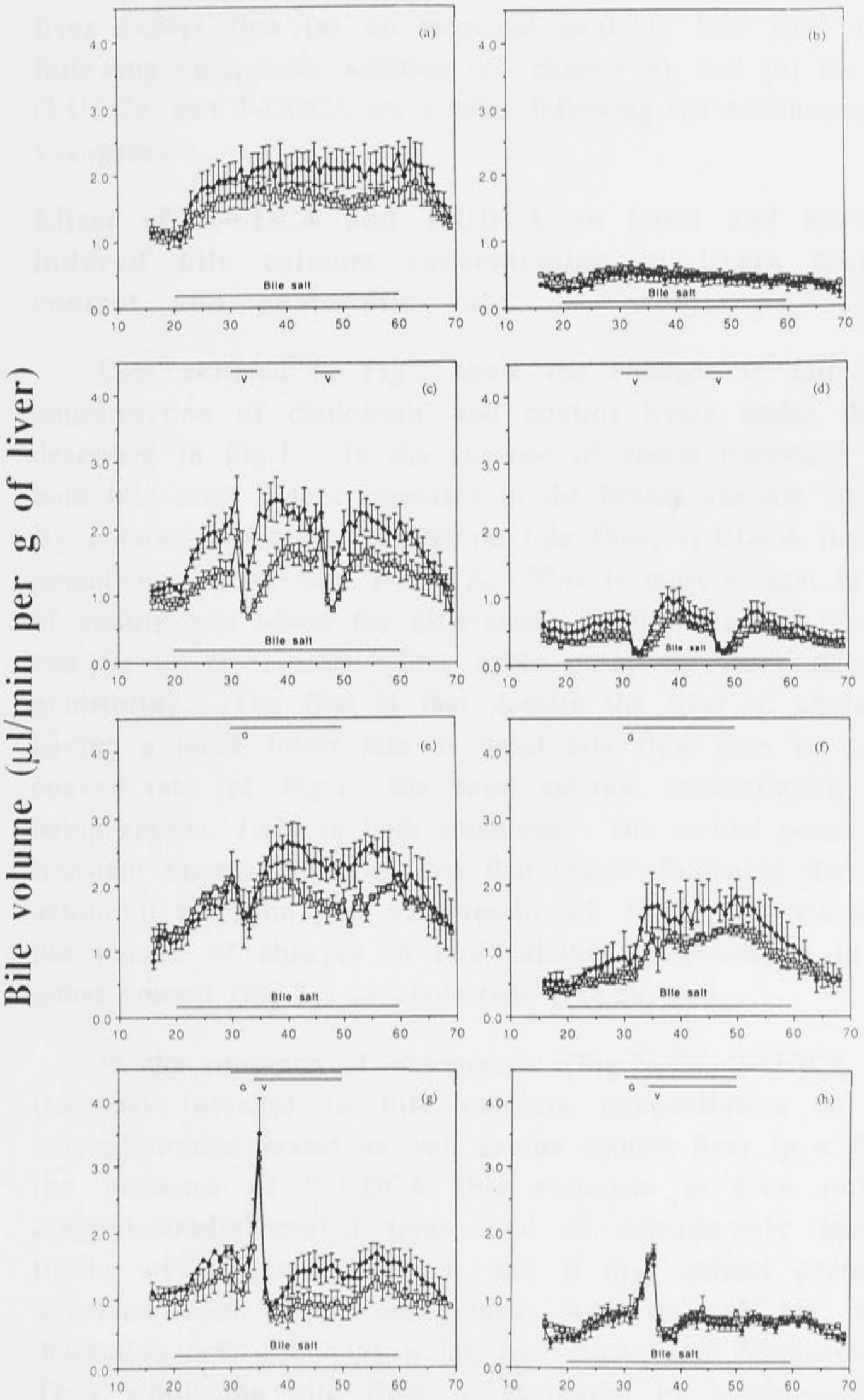
Data in Fig.1a show that both G-UDCA (as shown in Hamada et al., 1992b) and T-UDCA promote bile flow virtually from the time they are admitted to the liver of control rats. An increase of approx. 100% is induced within 5min and remains for as long as the bile acids are infused. The low rate of bile flow in the liver of cholestatic rats is only slightly but significantly increased ($p < 0.002$) by acute administration of the bile acids (Fig.1b).

The transient increase in bile flow in the control liver (Fig.1c) seen within a minute of administering vasopressin alone to control rats (see e.g. Hamada et al., 1992a; Nathanson et al., 1992a), is not significantly affected by either of the bile acids. This increase is absent in the cholestatic liver (Fig.1d); the characteristic subsequent cholestatic action of vasopressin otherwise is maintained. The early transient cholestatic action of glucagon (Hamada et al., 1992a; Bygrave et al., 1994) is unaffected by the bile acids in the control liver (Fig.1e), while the subsequent choleretic effect of glucagon is potentiated to a greater extent by T-UDCA than by G-UDCA ($p < 0.0001$). In the cholestatic liver (Fig.1f), the pattern of the bile flow response is maintained by both bile acids (see chapter 4), but again the flow of bile induced by glucagon is increased to a greater extent by T-UDCA ($p < 0.0001$) than by G-UDCA. This shows that the glucagon-induced choleretic response is more sensitive to T-UDCA than to G-UDCA in both the ethynylestradiol-treated and control animals.

Figs.1g and 1h show the effects of the two bile acids on the synergistic action of glucagon and vasopressin. With control liver (Fig.1g) the peak height attained immediately following vasopressin infusion is similar in the presence of each of the bile acids. Subsequently the two bile acids have distinctly different actions; in the presence of G-UDCA (but not T-UDCA) bile flow is suppressed for as long as the hormones are infused, a pattern similar to that seen without bile acids (Hamada et al., 1992a). However, in the presence of T-UDCA the bile flow rate increases substantially ($p < 0.0001$ vs G-UDCA) within 5-6 minutes following a brief decrease.

Fig.1. Effects of G-UDCA and T-UDCA on basal and hormone-induced bile flow in the perfused liver of control and cholestatic rats.

Livers from control rats (left-hand column) or those made cholestatic with ethynylestradiol as indicated in the experimental section (right-hand column) were perfused with Krebs-Henseleit bicarbonate medium containing 1.3mM Ca^{2+}. After a pre-perfusion period of 20min, 100µM G-UDCA (open symbols) or 100µM T-UDCA (closed symbols) was infused for 40min as indicated by the horizontal line. The responses observed in the absence of hormones are shown in (a) and (b). At 30min, 10min following the start of the infusion of the bile acids, 2 pulses of 10nM vasopressin (V) (c,d), a single pulse of 10nM glucagon (G) (e,f) or a combination of both hormones (g,h) were infused for the times indicated. For further details see the experimental section. The data are the means ± S.E.M. of three to six independent experiments. Data for G-UDCA in Fig.1a, 1c and 1g are from Hamada et al. (1992b) and are shown for comparison with those obtained with T-UDCA.



Bile volume (μl/min per g of liver)

Perfusion time (min)

The two bile acids induced similar responses in cholestatic liver (Fig.1h) following the synergistic action of glucagon and vasopressin. Their actions were similar to those observed in control liver except that (a) no transient peak in bile flow is induced following vasopressin addition (cf. chapter 4), and (b) the effects of G-UDCA and T-UDCA are similar following the attenuating action of vasopressin.

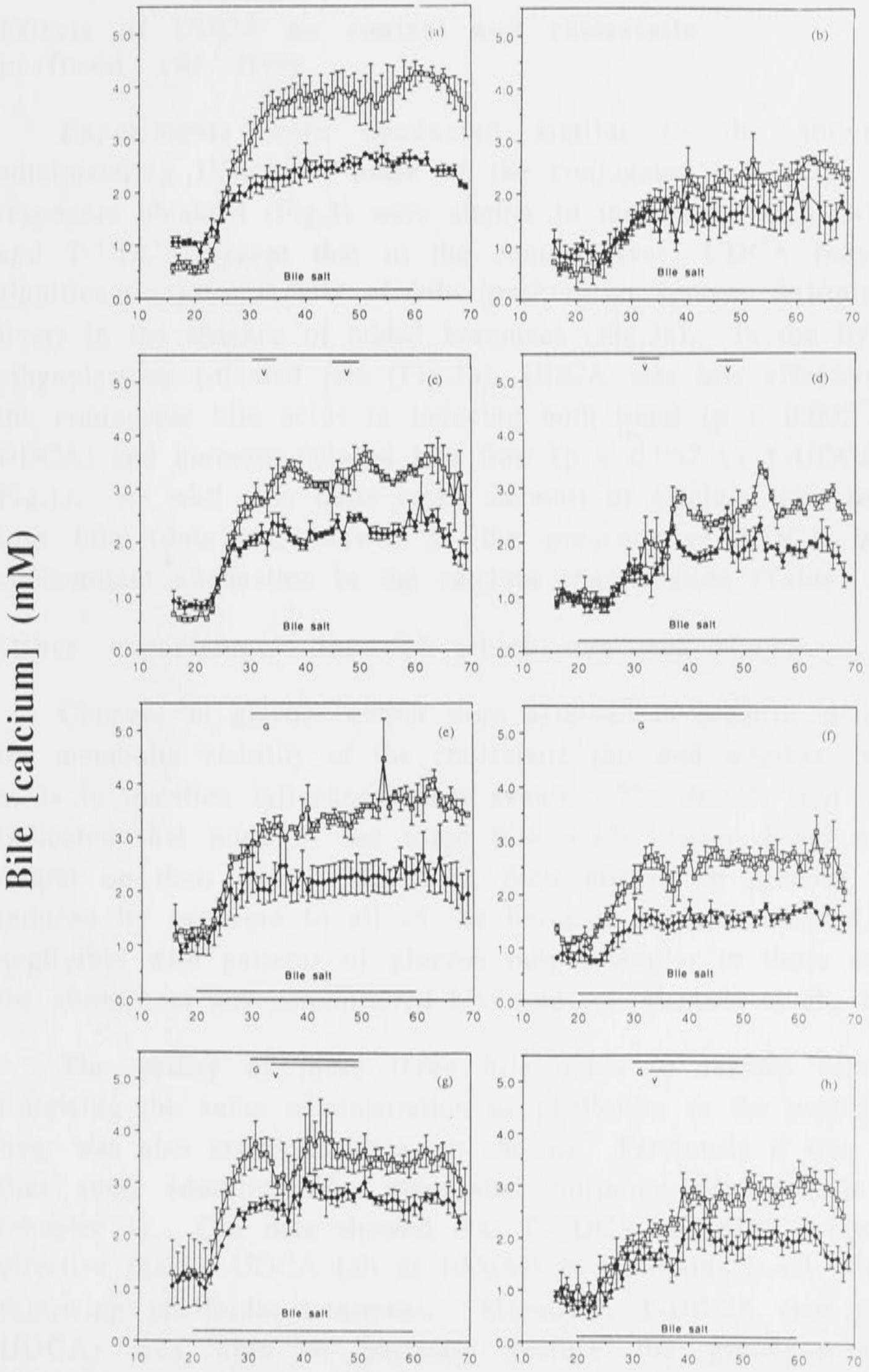
Effect of G-UDCA and T-UDCA on basal and hormone-induced bile calcium concentration in livers from control and cholestatic rats

Data collated in Fig.2 show the changes in biliary calcium concentration of cholestatic and control livers under the regimes described in Fig.1. In the absence of added hormones (Fig.2a,b), both bile acids induce increases in the biliary calcium concentration. By contrast with their effects on bile flow, G-UDCA has a greater potentiating effect than T-UDCA. This is most evident in the livers of control rats where the difference in biliary calcium concentration can be up to 1-fold. Two other points in these data also are noteworthy. The first is that despite the liver of cholestatic rats having a much lower rate of basal bile flow than in the liver of control rats (cf. Fig.1), the basal calcium concentration is similar, being approx. 1mM in both situations. The second point is that the transient increase in bile flow that occurs following the synergistic action of glucagon plus vasopressin (cf. Fig.1g), is not reflected in the pattern of changes in bile calcium concentration in livers of either control (Fig.2g) or cholestatic (Fig.2h) rats.

In the presence of vasopressin (Fig.2c,d), G-UDCA induces a transient increase in bile calcium concentration in both the ethynylestradiol-treated as well as the control liver ($p < 0.001$). In the presence of T-UDCA this response is seen only in the ethynylestradiol-treated liver, and is considerably smaller. The timing of the event is such that it may reflect event(s) which occurred soon after vasopressin was infused but which are detectable only following a lag (see Hacki and Paumgartner, 1973). Thus while the bile flow is decreased by vasopressin infusion (Fig.1c,d), calcium continues to be secreted into the bile, and this is detectable after it has travelled through the biliary tree.

Fig.2. Changes in biliary calcium concentration in the perfused liver of control and cholestatic rats following the administration of G-UDCA and T-UDCA.

The experimental system was exactly as described for Fig.1. The calcium concentration was determined by atomic absorption spectroscopy of the bile samples (Fig.1) as described in the experimental section. Open symbols are G-UDCA; closed symbols are T-UDCA. Data are the means $\pm$ S.E.M. of three to six independent experiments.



Bile [calcium] (mM)

Perfusion time (min)

When glucagon is administered to the cholestatic rat (Fig.2f,h), minimal changes in biliary calcium concentrations occur, but when vasopressin is added in the presence of glucagon (Fig.2h), there is a significant and sustained increase in bile calcium concentration.

Effects of UDCA on control and cholestatic perfused rat liver

Experiments were conducted similar to the above but administering UDCA in place of the conjugated bile acids. The responses obtained (Fig.3) were similar to those seen with G-UDCA and T-UDCA except that in the control livers UDCA induced a significantly greater flow of bile (peaking to approx. 2µl/min/g of liver) in the absence of added hormones (Fig.3a). In the livers of ethynylestradiol-treated rats (Fig.3b), UDCA was less effective than the conjugated bile acids in inducing both basal ($p < 0.002$ vs T-UDCA) and hormone-induced bile flow ($p < 0.067$ vs T-UDCA) (cf. Fig.1). As well only quite small amounts of calcium were secreted into bile (data not shown) in the presence of UDCA with a concomitant attenuation in the calcium concentration (Table).

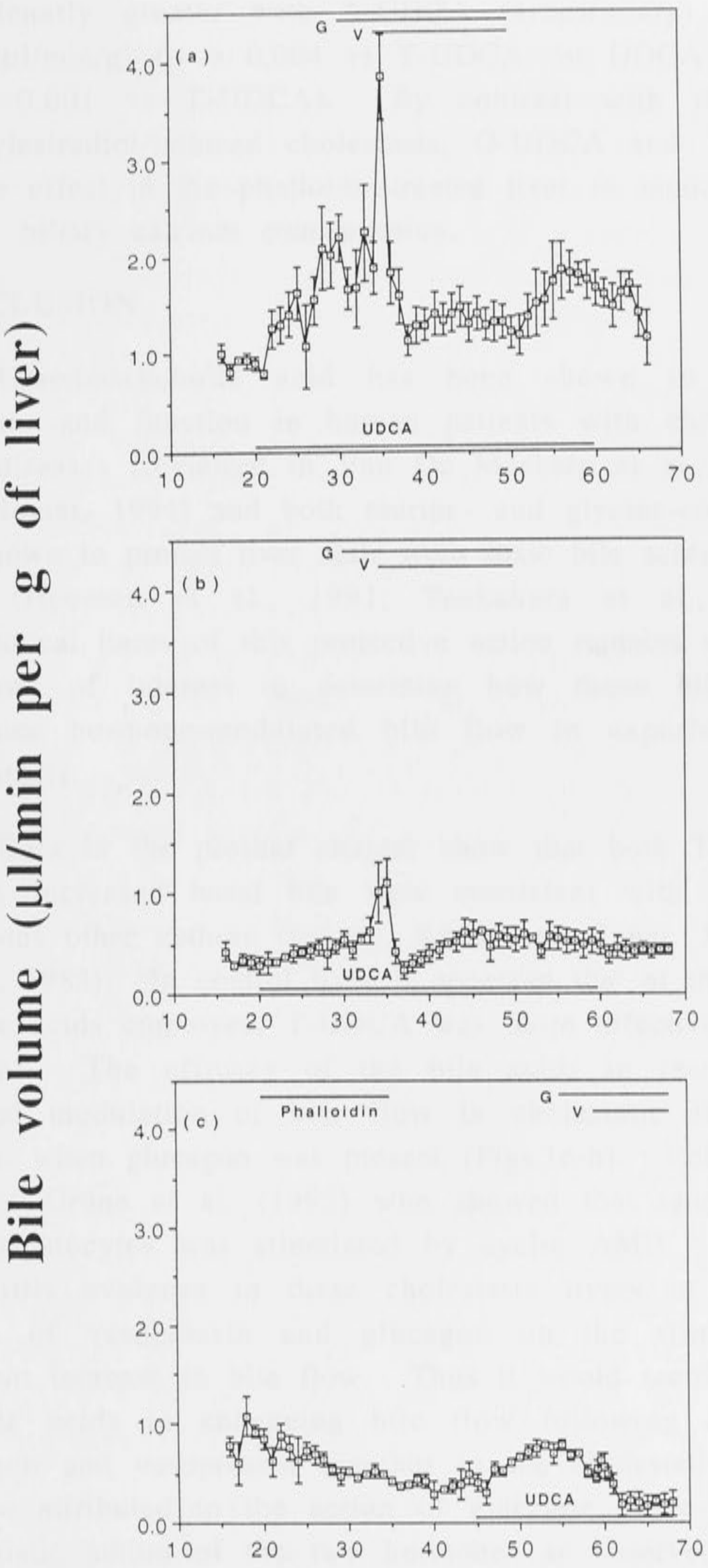
Other experiments data of which are not shown

Changes in glucose output were assessed in order to determine the metabolic viability of the cholestatic rats and whether the bile acids in question influenced these events. The results (not shown) indicated that none of the three bile acids altered basal glucose output on their own. Moreover, their effects on glucose output induced by exposure to all of the hormone regimes adopted, were negligible with patterns of glucose output similar to those seen in the absence of any administered bile acid (cf. Hamada et al., 1992a).

The ability of these three bile acids to restore bile flow following the acute administration of phalloidin to the perfused rat liver was also examined (data not shown). Previously it was shown that such administration attenuated hormone-induced bile flow (chapter 4). Our data showed that T-UDCA and UDCA are more effective than G-UDCA (all at 100µM) in promoting basal bile flow following phalloidin treatment. Moreover, T-UDCA (but not G-UDCA) was able to partially restore the glucagon-induced cholestasis as well as the vasopressin-induced increase in bile flow. The peak height from the pre-vasopressin baseline was

Fig.3. Influence of UDCA on glucagon-plus vasopressin-induced changes to bile flow in the perfused liver of control rats and those made cholestatic with ethynylestradiol or phalloidin.

Livers from control rats (a) or those made cholestatic in vivo with ethynylestradiol as indicated in the experimental section (b) or those made cholestatic in vitro with phalloidin (c) were perfused with Krebs-Henseleit bicarbonate medium containing 1.3mM Ca^{2+}. After a pre-perfusion period of 20min, 100µM UDCA was infused for 40min as indicated by the horizontal line. Where indicated vasopressin (V), glucagon (G) (each at 10nM) were infused for the times indicated. For further details see the experimental section. The data are the means ± S.E.M. of three to four independent experiments.



Bile volume (μl/min per g of liver)

Perfusion time (min)

significantly greater with T-UDCA (1.2µl/min/g) than G-UDCA (0.433µl/min/g) ($p = 0.004$ vs T-UDCA) or UDCA (0.168µl/min/g) ($p < 0.001$ vs T-UDCA). By contrast with their effects on ethynylestradiol-induced cholestasis, G-UDCA and T-UDCA had a similar effect in the phalloidin-treated liver in inducing an increase in the biliary calcium concentration.

CONCLUSION

Ursodeoxycholic acid has been shown to improve liver structure and function in human patients with chronic cholestatic liver diseases (reviewed in Van De Meeberg et al., 1993; Queneau and Montet, 1994) and both taurine- and glycine-conjugated UDCA are known to protect liver cells from toxic bile acids in vivo and in vitro (Heuman et al., 1991; Tsukahara et al., 1993). The biochemical bases of this protective action remains unclear. It was therefore of interest to determine how these bile acids would influence hormone-modulated bile flow in experimentally-induced cholestasis.

Data in the present chapter show that both T-UDCA and G-UDCA increased basal bile flow consistent with the findings of numerous other authors (see eg. Kitani and Kanai, 1982; Kanai and Kitani, 1983). In control liver it appeared that at the concentrations of bile acids employed, T-UDCA was more effective than G-UDCA (Fig.1a). The efficacy of the bile acids in restoring hormone-induced modulation of bile flow in cholestatic livers was more evident when glucagon was present (Figs.1c-h). Relevant to this is data of Grüne et al. (1993) who showed that taurocholate uptake into hepatocytes was stimulated by cyclic AMP. Moreover there was little evidence in these cholestatic livers of the synergistic action of vasopressin and glucagon on the stimulation of the transient increase in bile flow. Thus it would seem that the action of bile acids in enhancing bile flow following the infusion of glucagon and vasopressin together in the cholestatic liver (Fig.1h) can be attributed to the action of glucagon alone and not to the synergistic action of the two hormones as observed in control rat liver. It is evident however that despite the absence of synergistic enhancement of bile flow, vasopressin still is able to induce an immediate cessation of glucagon-enhanced bile flow in the cholestatic animal (cf. Fig.1g and 1h).

The relative abilities of the two conjugated bile acids to promote bile flow contrasted with their relative abilities to promote calcium flow into bile. In this latter aspect, G-UDCA was more potent than T-UDCA (Fig.2). Consequently a clear difference exists between the biliary calcium concentration induced by the bile acids in both control and cholestatic livers. Despite this, small but significant differences in hormone-induced changes in biliary calcium concentrations are evident (Figs.2c-h).

By comparison with their effects on bile flow and bile calcium, none of the bile acids had any significant effect on glycogenolysis, oxygen uptake or perfusate Ca^{2+} (data for the latter three were not shown), other than the relatively minor effects described in (Hamada et al., 1992b) involving G-UDCA. It is well known that these events are stimulated by Ca^{2+}-mobilising agonists (Reinhart et al., 1982a,b; Altin and Bygrave, 1986; Bygrave and Benedetti, 1993; Grüne et al., 1993). The extent to which changes in extracellular or intracellular Ca^{2+} are crucial in the mechanisms by which the bile acids are able to restore the hormone-modulated bile flow in cholestatic liver is unclear. In a previous study involving a cholestatic bile acid (taurochenodeoxycholic acid, T-CDCA) (Hamada et al., 1992b) significant basal as well as hormone-induced changes in each of these parameters was observed.

These experiments do not reveal information about the source of the biliary calcium. Cummings and Hofmann (Cummings and Hofmann, 1984), in an extensive study defining the origins and physiologic determinants of biliary calcium secretion in the dog, concluded that biliary calcium output in the dog has two components, canalicular and ductular. Their work indicated that most biliary calcium originates at the canaliculus and is bile acid-dependent and that although the biliary ductule is permeable to calcium, the effect of ductular modification of bile flow on biliary calcium output is small.

As shown in the Table, the differences in the changes in biliary calcium concentration and bile flow rate are much greater between the bile acids than in the changes in calcium secretion rate into bile. Furthermore, the calcium secretion rate induced by UDCA is considerably less than that induced by T-UDCA and G-UDCA. Data published elsewhere show a substantial range of variation.

Table Comparison of the effects of UDCA, T-UDCA and G-UDCA on biliary secretory parameters in control rat liver

	Bile Acid		
Measurement made*	UDCA	T-UDCA	G-UDCA
Increase in bile secretion (μl/min/g liver)	1.871 ± 0.179	0.582 ± 0.033	0.250 ± 0.055
Increase in rate of calcium release into bile (nmol/min/g liver)	1.132 ± 0.147	2.729 ± 0.089	3.197 ± 0.159
Increase in bile [calcium] (mM)	0.209 ± 0.045	1.216 ± 0.059	2.254 ± 0.082

* For the purposes of this comparison, each measurement represents the net values (± S.E.M.) of the difference between the basal perfusion times (15 to 20 min) and those after addition of the bile salt (26 to 30 min) in Figs. 1g, 2g and 3a.

Generally though they show a similar pattern; that is, less UDCA is secreted into the bile than either G-UDCA or T-UDCA following infusion of the respective bile acids, although the choleretic effect is greater with UDCA (Scharschmidt and Lake, 1989; Roda et al., 1989; Anwer, 1992).

Considerable differences were observed in the effects of the bile acids used in this study in the two models of cholestasis studied. Where phalloidin was used, UDCA induced the greatest increase in bile flow, but T-UDCA induced the greatest increase in glucagon and vasopressin-induced peak size. Tsukahara et al (1993) concluded on the basis of studies on T-CDCA-induced cholestasis in vivo that T-UDCA had a more significant hepato-protective effect, despite the greater choleretic effect of UDCA. The present results indicate that while none of the bile acids studied significantly altered the pattern of the hormonal responses of ethynylestradiol-treated rat liver, the effect of T-UDCA was to noticeably increase the height of the transient peak induced by vasopressin in the presence of glucagon in the phalloidin-treated liver. Thus the specificity in the beneficial effects of the bile acids in cholestatic disease states may be a function not only of bile acid structure but also the underlying mechanism by which the cholestasis has been induced.

Section F

GENERAL DISCUSSION

General Discussion

The ubiquitous role of Ca^{2+} in cellular signalling is universally acknowledged. The activity of many enzymic cascades and other proteins in cells are modulated by changes in intracellular Ca^{2+} concentration; thus the mobilization of Ca^{2+} provides a mechanism for rapid stimulation of physiological responses. The regulated opening of Ca^{2+} channels at the endoplasmic reticulum as well as on the plasma membrane following the interaction of hormones with their cell-surface receptors is the basic mechanism by which many hormones elicit their cellular responses. Since the extracellular medium contains an unlimited source of Ca^{2+} (Barritt, 1992), influx of Ca^{2+} across the plasma membrane from the extracellular medium is essential in order to sustain any Ca^{2+}-mediated responses, as well as to replenish depleted intracellular stores following release of the ion from these upon stimulation with hormones. The precise mechanism by which the opening of Ca^{2+} channels, especially those on the plasma membrane, are regulated has so far proved to be elusive. However, it has been recognized for some time that influx of Ca^{2+} across the plasma membrane is considerably facilitated by cross-talk between the Ca^{2+}-mobilizing and cyclic AMP-generating hormones.

As indicated in the Introductory Review, the role of Ca^{2+} in the flow of bile is reflected by the observation that removal of the ion from the extracellular medium results in the cessation of bile flow (Graf, 1975; Owen, 1977; Reichen et al., 1985). The role of Ca^{2+} mobilization in the modulation of the flow of bile is indicated by the fact that the Ca^{2+}-mobilizing hormones are able to modulate such flow (Graf, 1975; Krell, 1985; Hamada et al., 1992a; Nathanson et al., 1992a). Moreover, this action is modified by the cross-talk mentioned above (Hamada et al., 1992a; Bygrave et al., 1994). The work described in this thesis has resulted in advances in knowledge on the mechanism by which cross-talk between the IP_3- and cyclic AMP-signalling systems modulates Ca^{2+} mobilization and bile flow, resulting in further insights into the relationship between these events.

The perfused liver system

Ca^{2+} fluxes have been studied in both the perfused rat liver (see e.g. Altin and Bygrave, 1988; Bygrave and Benedetti, 1993 for a review) and in hepatocytes, and the information obtained from these seemingly disparate systems generally is complementary. Consistent with this fact are data in this work showing the concentrations at which vasopressin alone induces Ca^{2+} release from the perfused rat liver (see Fig.2, Introductory Review). As indicated in the Introductory Review, a major advantage of the perfused liver in studies of cellular Ca^{2+} fluxes is that the temporal relationship between net efflux and net influx, and their relationship to other metabolic events such as bile flow in response to agonist stimulation, can be quantitatively analysed.

In a recent study examining the responses of bile flow and Ca^{2+} mobilization to the rapid action of vasopressin, Nathanson et al. (1992) concluded that increases in cytoplasmic Ca^{2+} from either internal or external sources, lead to attenuated bile flow. However, their conclusion was based on matching data from experiments carried out with the perfused rat liver (in which bile flow was measured), and with isolated liver cells (in which changes in intracellular Ca^{2+} was measured). A relevant attribute of the perfused rat liver system employed in this laboratory is the ability to measure small changes in perfusate Ca^{2+} that reflect either or both Ca^{2+} inflow and Ca^{2+} outflow and hence plasma membrane Ca^{2+}-cycling (Reinhart et al., 1984a; Altin and Bygrave, 1985). Together with measurements of very rapid changes in bile flow, this represents a further advance in the ability to directly correlate changes in plasma membrane Ca^{2+} fluxes with those in bile flow.

Insights into the mechanism of Ca^{2+} influx

- the role of Ca^{2+}-mobilizing agonists

An overview of the major mechanisms presently considered to be involved in the stimulation of Ca^{2+} fluxes by Ca^{2+}-mobilizing agonists has been presented in the Introductory Review (Part 1, Section (D) (III)). Further clues on the relationship between efflux and influx events are presented in this work, as well as the way in which cross-talk between the signalling systems modifies these

responses. Together they provide further insights into the mechanisms involved in the regulation of these events.

The extent to which Ca^{2+} is released from intracellular Ca^{2+} stores is dependent on IP_3 production (Thomas et al., 1984; Charest et al., 1985; Lynch et al., 1985). The quantal nature of this release is well known (see e.g. Muallem et al., 1989; Bootman, 1994; Taylor, 1995), but the mechanism is unclear, although a number of proposals have been suggested (see, e.g. Bootman, 1994). The data in Chapter 3 indicate that a threshold concentration for the release of Ca^{2+} from the liver occurs at approximately 0.1nM vasopressin, with no release observed at 0.05nM. The latter point was confirmed by the absence of changes in glucose release as well as bile flow (data not shown). Interestingly, a similar threshold has been detected with oscillations in intracellular Ca^{2+} concentrations in hepatocytes (Sanchez-Bueno et al., 1993). It has been recognised for some time that there is an 'explosive' increase in the intracellular Ca^{2+} concentration upon the IP_3 concentration reaching a particular threshold (Miledi and Parker, 1989; Petersen and Dupont, 1994). While the nature of the 'switch' for this event is unknown, an important possibility is the role of the synergism between IP_3 and Ca^{2+} on the activity of the IP_3 receptor (Striggow and Bohnensack, 1994; Bootman et al., 1995).

The threshold at which vasopressin begins to stimulate net influx of Ca^{2+} at 1nM is considerably higher than that for net efflux (Fig.1, Chapter 3). Thus for net influx to occur, a higher rate of receptor occupancy is required, associated with which is an increased rate of IP_3 production. Whether the primary mechanism by which influx occurs is directly related to an interaction of the occupied vasopressin receptor and the Ca^{2+} channel (see e.g. Felder et al., 1994), or whether the influx is secondary to the increased IP_3 production directly (see e.g. Striggow and Bohnensack, 1994), or the subsequent depletion of the endoplasmic reticular Ca^{2+} stores (capacitative Ca^{2+} influx) (Putney, 1990; Fasolato et al., 1994), requires examination.

The fact that efflux always precedes net influx of Ca^{2+} (see also e.g. Toescu and Petersen, 1995; but see also Lupu-Meiri et al., 1994), and that a larger amount of Ca^{2+} is released at the higher concentrations of the agonist, suggests that depletion of the

intracellular Ca^{2+} stores is involved in this event. By this scheme, the state of Ca^{2+} depletion of the endoplasmic reticulum is communicated to the plasma membrane either through an intracellular messenger molecule (such as an influx factor [see, e.g. Parekh et al., 1993; Radriamampita and Tsien, 1993, 1995; but see also Bird et al., 1995; Gilon et al., 1995]), or a direct connection exists between the endoplasmic reticulum and the plasma membrane (Irvine, 1990), resulting in influx. Variations around this general idea are plentiful, with different factors suggested (see the Introductory Review). The inactivation of the influx involves both store-dependent (Jacob, 1990; Montero et al., 1992; Dolmetsch and Lewis, 1994) as well as store-independent pathways (Zweifach and Lewis, 1995).

However, the extent of emptying of the intracellular stores, as reflected by the amount of Ca^{2+} released, does not always correlate with spontaneous influx of Ca^{2+}. For example, phenylephrine alone induces a large efflux (see Fig.1 Chapter 1, as well as Reinhart et al., 1982a, 1984a,b; Altin and Bygrave, 1985), but despite the more extensive depletion of the intracellular stores than with high concentrations of vasopressin, no spontaneous influx of Ca^{2+} is induced. Thus there are subtle differences in the mechanism by which Ca^{2+} mobilization is regulated by these agonists (see e.g. Toescu, 1995). This is further indicated by the different patterns of oscillations in intracellular Ca^{2+} concentration induced by these agonists in hepatocytes, despite both acting through IP_3 generation (see e.g. Rooney et al., 1989; Sanchez-Bueno et al., 1993; Toescu, 1995).

The second messengers generated could be directly involved in the stimulation of Ca^{2+} influx. Evidence has been presented for a stimulation of Ca^{2+} influx by the action of IP_3 directly (Striggow and Bohnensack, 1994), an action which would be dependent on the presence of IP_3-gated channels on the plasma membrane (see e.g. Lückhoff and Clapham, 1992), channels gated by a metabolite of IP_3, such as IP_4, or a further undetermined function of this messenger. The fact that the maximum rate of Ca^{2+} released with low extracellular Ca^{2+} concentrations are similar with phenylephrine and vasopressin (Altin and Bygrave, 1985), indicates that both these agents release Ca^{2+} from the intracellular Ca^{2+} stores with a similar potency. However, adrenergic agonists have

been shown to generate less IP_3 than vasopressin (Creba et al., 1983; Charest et al., 1985; Lynch et al., 1985). Therefore, while maximal release of Ca^{2+} is induced in response to this stimulation of IP_3, the potential role of the extra IP_3 produced needs to be borne in mind.

It is noteworthy that the response observed with low concentrations of vasopressin (Fig.1b, Chapter 3) resembles that seen with phenylephrine where, following the stimulation of Ca^{2+} release, influx is observed only subsequent to the removal of the hormone (Reinhart et al., 1982a, 1984a; Altin and Bygrave, 1985), although the magnitude of the response is much smaller. This similarity in the pattern of the responses points to a stimulation of the Ca^{2+}-cycling rate (Reinhart et al., 1984a) even with the low concentrations of vasopressin. This is further suggested by the sustained increase in glucose release despite the absence of net Ca^{2+} flux changes following the efflux (data not shown), as observed with phenylephrine (Reinhart et al., 1984b). Thus an increased flux of Ca^{2+} through the inflow channels is probably stimulated even under these conditions of lower IP_3 production, despite this not being observed as net influx.

Interactions at the level of the plasma membrane between the receptor and the Ca^{2+} channel have been suggested as a mechanism for the stimulation of influx by these agonists. Dasso and Taylor (1994) have shown that one of the differences in the activity of vasopressin and phenylephrine potentially lies in the differences in the interaction of these agonists with G-proteins at the plasma membrane. Their work indicated that either both agonists share the same G-protein pool, but have different affinities to these, or alternatively, vasopressin has access to another pool of G-proteins, which is not as accessible to phenylephrine. This could explain the differences in the effects observed in response to these agonists (Bygrave and Bennedetti, 1993; Bygrave and Roberts, 1995). By this scheme, a pool of G-proteins could be involved with the interaction between the receptor and Ca^{2+} channel, for which the vasopressin receptor would have a greater affinity than phenylephrine. As indicated earlier, both these agents probably release Ca^{2+} with a similar potency at maximally-stimulating concentrations. Thus it is suggested that the G-protein pools involved in transducing the influx and for the stimulation of

phospholipase C, are separate. The affinity of the vasopressin-receptor complex for this G-protein pool would be lower than for the pool involved in the stimulation of phospholipase C, or alternatively, there is a lower number of these. This would explain the differences between the responses observed at the different concentrations of vasopressin, as well as those observed with phenylephrine.

The transient nature of influx at the higher concentrations of vasopressin, despite continuing receptor occupation, argues against a direct coupling to the channel as being the only mechanism involved, unless a rapid desensitisation mechanism is operating. Thus it may be proposed that both depletion of the stores as well as an interaction between the receptor and the channel at the plasma membrane are involved in the stimulation of influx. In fact, it may be that separate mechanisms might be involved in stimulating the influx which is associated with the increased Ca^{2+}-cycling rate, and that which is observed as net influx under the experimental conditions employed. Whether the separate mechanisms might also involve separate Ca^{2+} inflow pathways still needs to be clarified.

The role of glucagon in vasopressin-induced Ca^{2+} influx

It has previously been shown in the perfused rat liver (Altin and Bygrave, 1986; Benedetti et al., 1989), in hepatocytes (Mauger and Claret, 1986; Poggioli et al., 1986; Burgess et al., 1991) that glucagon is able to synergistically enhance the influx induced by the Ca^{2+}-mobilizing agonists. In summary, the data in Chapters 1 and 3 indicate that the effects of glucagon on the actions of the Ca^{2+}-mobilizing agonists include decreased lag time to the start of the response (see also e.g. Kass et al., 1990), and an increase in the rate of influx and efflux, the acceleration in that rate and an increase in the duration of influx. Thus the predominant effect appears to be on the influx of Ca^{2+}. The mechanisms by which these effects might occur are now discussed.

In view of the observation that glucagon by itself stimulates a net release of Ca^{2+}, and that the release of Ca^{2+} from the endoplasmic reticular store by the Ca^{2+}-mobilizing agonists is enhanced by this hormone (Burgess et al., 1991; Hajnocky et al., 1993), it could be argued that sensitizing for influx by a greater

depletion of the stores (Kass et al., 1990, 1994b) could be part of the mechanism by which the influx is stimulated by the combination of glucagon and the Ca^{2+}-mobilizing hormones. The total amount of Ca^{2+} released by glucagon is small (see e.g. Bygrave and Benedetti, 1993), and generally less than that observed with the low concentrations of vasopressin alone, even at maximally stimulating (10nM) concentrations of glucagon. Thus the enhancement of efflux alone can probably only be considered to be the main mechanism involved in the stimulation of influx under these conditions if cross-talk promotes the release of Ca^{2+} from regions of the endoplasmic reticular store which are more sensitive or responsive to the depletion. The possibility of such heterogeneity in the endoplasmic reticular store has been suggested (see e.g. Shuttleworth, 1994). This possibility is further suggested by the observation that Ca^{2+} release induced by thapsigargin is enhanced in the presence of glucagon (Burgess, et al., 1991). The extent to which the release of Ca^{2+} from IP_3-sensitive and -insensitive Ca^{2+} stores (Kass et al., 1990; Sitia and Meldolesi, 1992; Gamberucci et al., 1995), or the transfer of the ion between these (Dawson, 1985; Gill et al., 1986; Putney and Bird, 1993a; Hajnoczky et al., 1994; Berven et al., 1995) are relevant to this question, is presently unclear.

In the presence of glucagon the efflux still always precedes the influx of Ca^{2+}. However, in the presence of glucagon, influx occurs even with a lower rate of Ca^{2+} release, and in fact there appears to be some degree of correlation between the rate of acceleration of the influx and the rate of acceleration of the efflux. This may point to the stimulation of a mechanism common to both release and influx, features apparent only at low concentrations of the hormones.

In view of the circumstantial evidence discussed earlier pointing towards the involvement of G-proteins in these events (see e.g. Dasso & Taylor, 1994), their regulation could involve cyclic AMP -mediated enhancement of the interaction between the receptor for the Ca^{2+}-mobilizing agonist and the Ca^{2+} channel. This possibility is suggested by the pattern by which the channels are opened in the presence of glucagon (see Fig.2-4, Chapter 3). Notable is the effect of glucagon on the influx induced by vasopressin, whereby the duration and maximal rate are increased in a concentration-

dependent manner. Additionally, the smaller total net efflux under these conditions can probably be explained by the earlier onset of the influx relative to the start of the (efflux) response, so that the rate of efflux which would occur in the absence of glucagon is not attained. This latter point is indicated also in Chapter 2 (Fig.1a) where total inhibition (by Ni^{2+}) of influx induced by glucagon and vasopressin results in a greater maximum rate as well as duration of efflux. Since efflux occurs subsequent to a G-protein-mediated cascade of events at the plasma membrane (see Introductory Review, Part 1, Section (D) (III)), any direct facilitation of this interaction would be expected to decrease the relative timing of these events.

The influx rate decays more rapidly following removal of the hormones (see Fig.3 and Fig.4, Chapter 3), indicating that receptor occupancy is necessary for the cross-talk mediated influx to occur. Thus the decrease in the timing of the influx relative to efflux provides further evidence that an interaction between the receptors involved and the Ca^{2+} channel is facilitated by glucagon. However, the role of depletion of intracellular Ca^{2+} stores in this scheme cannot be totally discounted either, especially since glucagon also enhances the release of Ca^{2+}. Additionally, enhancement of events at the level of second messengers cannot be eliminated since some studies have shown that higher IP_3 levels can be formed in the presence of cyclic AMP (e.g. Pittner and Fain, 1989 but see e.g. Creba et al., 1983; Burgess et al., 1991). It may be possible to distinguish between these possibilities with further studies involving direct modulation of components of these signalling pathways.

Various reports have shown that agents which deplete the endoplasmic reticular Ca^{2+} stores, stimulate influx of Ca^{2+} (Putney, 1990; Kass et al., 1992). Furthermore, the influx of Ca^{2+} by vasopressin may be enhanced following treatment with agents which deplete the endoplasmic reticular Ca^{2+} pool (such as BHQ and thapsigargin) (Kass et al., 1990, 1994a; data not shown). Thus further work would involve depleting the intracellular stores without IP_3. Additionally preliminary experiments have shown that in the presence of BHQ and thapsigargin, influx can be induced with low concentrations of glucagon and cyclic AMP (data not shown; T. Applegate, A. Karjalainen and F.L. Bygrave *in*

preparation). While Llopis et al. (1993) showed that some the release of Ca^{2+} in response to BHQ could be due to BHQ-mediated release of eicosanoids from the other cell types in the liver, the amount of Ca^{2+} taken up in these experiments was far greater than that with various eicosanoids added at unphysiologically high concentrations (Altin et al., 1987). Thus store depletion can interact synergistically with both signalling systems to induce influx. Since evidence exists for the involvement of multiple Ca^{2+} transport systems in Ca^{2+} influx (Barritt et al., 1981; Hughes et al., 1986; Altin and Bygrave, 1987; Barritt and Hughes, 1991; Llopis et al., 1992; Kass et al., 1994), it is possible that different populations of channels are involved in the different mechanisms discussed. Further research would need to be directed toward conditions where glucagon is able to induce influx in the absence of a Ca^{2+}-mobilizing agonist, such as in the presence of increased perfusate pH (Altin and Bygrave, 1987; Bygrave and Benedetti, 1993).

Further putative sites of interaction exist however. The suggestion that the enhanced influx results from a glucagon-mediated increase in the Ca^{2+} uptake-capacity of intracellular stores, such as the mitochondria (Morgan et al., 1983; Altin and Bygrave, 1986; Barritt and Hughes, 1991), or the endoplasmic reticulum (Banhegyi, et al., 1991) cannot be ruled out. Where the large influx of Ca^{2+} occurs in response to the cross-talk between the signalling systems the mitochondrial Ca^{2+} content increases substantially (Altin and Bygrave, 1986); whether this occurs simply in response to the increased influx of the ion remains to be determined. Recently it has been shown that energization of the mitochondria enhance the Ca^{2+}-oscillatory effects induced by IP_3 (Jouaville, et al., 1995). Furthermore, glucagon has been shown to increase the mitochondrial Ca^{2+} uptake induced by thapsigargin (Hoek et al., 1995). While the mechanism by which glucagon might promote the influx of Ca^{2+} into the mitochondria is not presently clear, it has been suggested that it could be related to its ability promote the release of Mg^{2+} from this organelle (Romani et al., 1991; Hoek et al., 1995), although a more direct effect is not ruled out.

While conflicting data exist for the role of an interaction of either IP_3 or its metabolite IP_4 (see e.g. Striggow and Bohnensack, 1994) with the plasma membrane in inducing influx, it is pertinent

to note that the activity of IP_3-kinase has been shown to be synergistically stimulated in hepatocytes (Biden et al., 1988).

Thus even in the presence of low concentrations of glucagon, low concentrations of vasopressin are able to stimulate influx of Ca^{2+}, despite minimal Ca^{2+} release. Therefore cross-talk between the signalling pathways presents a mechanism by which the cells can be sensitised to influx Ca^{2+}, enabling responses to be elicited even by physiological concentrations of agonists (Bygrave and Benedetti, 1993).

Ni^{2+} as a tool for modulating the influx of Ca^{2+} in the perfused rat liver

The relationship between the Ca^{2+} fluxes induced by cross-talk and its physiological consequences can be further examined utilising an inhibitor of Ca^{2+} influx. Ni^{2+} appeared to be an appropriate candidate to modulate Ca^{2+} entry in this work as many of the classical Ca^{2+} channel blockers are ineffective at low concentrations with this tissue (Mauger and Claret, 1988; Hughes and Barritt, 1989). Despite earlier characterization of the role of Ni^{2+} in hepatocytes (see e.g. Hughes and Barritt, 1989), there are no previous reports where the effects of Ni^{2+} on Ca^{2+} fluxes have been characterised in the perfused rat liver. As indicated in Chapter 2, (Fig.1a), Ni^{2+} inhibits Ca^{2+} influx in a concentration-dependent manner. While the half-maximum inhibition of the rate of influx is observed at approximately 200µM Ni^{2+}, the half-maximum inhibition of the total amount of Ca^{2+} taken up is approximately 25µM, due to the Ni^{2+} concentration-dependent rapid decay of the influx under these conditions. This suggests that the inhibition of the influx follows the binding of Ni^{2+} at a Ca^{2+}-binding site subsequent to the opening of the channel. Furthermore, since infusion of 500µM Ni^{2+} results in the almost total inhibition of Ca^{2+} influx in the presence of 1.3mM Ca^{2+}, the affinity of the Ca^{2+}-binding site in the channel for Ni^{2+} is considerably higher than for Ca^{2+}. Whether there is a single population of channels involved in the cross-talk-mediated stimulation of Ca^{2+} influx, as has been indicated in influx induced by the agonists alone (Fernando and Barritt, 1994b), will require a comparison of the sensitivity of the influx to inhibition by other, more specific, Ca^{2+} channel antagonists. Examples of these include some of the lanthanides,

such as gadolinium (Fernando and Barritt, 1994b), as well as SK&F96365 (Meritt et al., 1990; Fernando and Barritt, 1994b) and econazole (Villalobos et al., 1992; but see Sargeant et al., 1994). It is presently unclear whether the 'molecular entities' involved in the mechanism by which basal influx of Ca^{2+} occurs are the same as those involved with the hormone-mediated Ca^{2+} influx (Barritt and Hughes, 1991).

The relationship between Ca^{2+} fluxes and bile flow

A link between the mechanisms involved in inducing the flow of bile and Ca^{2+} fluxes in the liver is suggested by the considerable evidence discussed in the Introductory Review, and the modulatory effect of Ca^{2+} on many of the processes associated with the flow of bile. Various processes resulting from the action of the Ca^{2+}-mobilizing agonists as well as glucagon, have been implicated in bile flow events (see Introductory Review Fig.6a-c). These include (for vasopressin) the stimulation of the uptake of bile acids (Divald, 1994), stimulation of the transport of organic anions into the canaliculus (Roelofsen et al., 1991), stimulation of microfilament contraction (Oshio et al., 1985), as well as the stimulation of vesicular transport from the sinusoidal to the canalicular pole, and their exocytosis through Ca^{2+} mobilization (Beuers et al., 1993a). However, Ca^{2+} is also thought to increase permeability of the tight junctions (Hardison et al., 1988). Additionally, increased activity of protein kinase C as a result of vasopressin action is thought to antagonize some of the effects of Ca^{2+} (Nathanson and Boyer, 1991; Corasanti et al., 1989; Bruck et al., 1994), while co-stimulating others (see Fig.6, Introductory Review). Furthermore, cyclic AMP is thought to stimulate the activity of some canalicular transporters (see e.g. Boyer et al., 1992), although its effect on those transporters involved in the transport of bile acids remains to be clarified. Additionally, cyclic AMP has been shown to stimulate the uptake of bile acids (Grüne et al., 1993), and the transfer of vesicles from the sinusoidal pole to the canaliculus (Lowe et al., 1988; Hayakawa et al., 1990, Hoshino et al., 1993). The relative importance of many of these processes, as well as the extent to which they are affected by Ca^{2+} mobilization is presently unclear. However this work has provided further clues on the relationship between hormone action and bile flow.

Effect of Ni^{2+} on the basal bile flow rate

The basal bile flow rate has been shown to be inhibited upon removal of extracellular Ca^{2+} (Graf, 1975; Owen, 1977; Reichen et al., 1985). As suggested in the Introductory Review, this could be due to the decreased rate of Ca^{2+}-cycling across the plasma membrane under these conditions (Reinhart et al., 1984a), associated with some possible degree of depletion of the intracellular Ca^{2+} stores. Evidence for this includes the observation that there is a decrease in the bile flow rate which occurs transiently soon after the start of Ni^{2+} infusion (Fig.1, Chapter 2), and that in the presence of 500µM Ni^{2+}, this is considerably more sustained. In fact in data not shown, infusion of 1mM Ni^{2+} resulted in an even more substantial decrease in bile flow during a 20 minute infusion, which recovers upon removal of the ion. Few direct effects of Ni^{2+} on any of the intracellular processes involved in the flow of bile have been shown, although disruption of the intermediate filament network occurs in cultured hepatocytes which have been incubated in the presence of high concentrations of Ni^{2+} for 24 hours (Kawahara et al., 1990).

Thus a relevant question in this context is whether Ni^{2+} actually enters the liver cell. Data obtained with other cell types including platelets (Hallam and Rink, 1985), keratinocytes (Jones and Sharpe, 1994), umbilical cord cells (Hallam et al.,1988) and with hepatocytes (Nathanson et al., (1992a,b) indicate that Ni^{2+} does not enter cells under various experimental conditions, even under conditions where the ion is present at high extracellular concentrations. On the other hand Crofts and Barritt (1990) concluded that the ion can enter hepatocytes following vasopressin stimulation, albeit at a significantly slower rate than that observed with other divalent ions. However in view of the less invasive nature of the perfused liver technique, minimal entry of the ion into the cells would be expected under these experimental conditions, and therefore a direct effect of (what would be intracellular) Ni^{2+}, although not totally excluded, is probably unlikely.

At the same time some of the data are consistent with either, or both, depletion of intracellular Ca^{2+} stores, as well as a decrease in Ca^{2+}-cycling rate: see for example (1) the delay before the onset

of the vasopressin-induced efflux (approx. 18s relative to the control, see 500μM Ni^{2+} curve in Chapter 2, Fig.2a), and (2) the decreased rate, as well as the magnitude of this efflux relative to vasopressin alone (see Fig.1 in Chapter 1). Thus a link between the state of filling of intracellular Ca^{2+} stores or Ca^{2+}-cycling rate and basal bile flow is strongly suggested. Alternative explanations for these events, however, cannot be entirely excluded.

Modulation of bile flow by signalling cross-talk

The synergistic stimulation of Ca^{2+} influx by cross-talk between glucagon and vasopressin is reflected in the short-term stimulation of bile flow (Hamada et al., 1992a; Bygrave et al., 1994). Furthermore, a similar cross-talk-mediated decrease in the lag time from the start of hormone infusion to the start of the Ca^{2+} fluxes is also observed with bile flow. Thus Ca^{2+} fluxes are strongly implicated in bile flow events. As indicated in the Introductory Review, changes in Ca^{2+} fluxes clearly result in modifications in bile flow. Furthermore, data from Chapter 1 indicate that the extent to which influx of Ca^{2+} is stimulated is reflected in the extent to which the flow of bile is stimulated.

Various studies have shown that increases in bile flow induced by the Ca^{2+}-mobilizing agonists are closely associated with a decrease in that rate (see, e.g. Graf, 1975; Krell, 1985; Hamada et al., 1992a; Nathanson et al., 1992a). An analysis of the response induced by glucagon plus vasopressin reveals that the volume of bile in the 'peak' (integrated from base-line to base-line) was approx. 16% of the total canalicular volume of approx. 5μl/g of liver (Barber-Riley, 1963). However the total amount of bile secreted in this early phase was less than 6% of the subsequent deficit in bile production. Since this would appear to be a small proportion only of the total deficit, it seems likely that the full explanation of the hormone-induced changes in bile flow is not contraction followed by dilation of the biliary tree. A similar argument was put recently by others (Nathanson et al., 1992a), and sudden collapse of the canaliculus has been suggested as an explanation.

Large, non-specific increases in cytoplasmic Ca^{2+}, such as those that have been induced by the ionophore A23187, or the

inhibitors of SERCA ATPases (Llopis et al., 1991; Nathanson et al., 1992a; Bruck et al., 1994; but see also Farrell et al., 1990) tend to decrease bile flow. Since there are indications that Ca^{2+} may inhibit the polymerization of actin (see e.g. Janmey et al., 1994), the resultant disruption of the microfilament network may be part of the explanation for this effect. The maximum decrease in bile flow rate is approximately 60% with all the treatments mentioned above, as well as under all conditions described in this work. While influx of Ca^{2+} is implicated in this event, since it is inhibited by Ni^{2+} infusion in a concentration dependent manner, it is worth noting that some influx occurs even in the presence of 250μM Ni^{2+}. Thus a role for Ca^{2+} that is related to its localization within the cell is indicated. However, the increase in bile flow rate always precedes the decrease, and the timing of this event suggests that Ca^{2+} mobilization could be involved in both these processes. Data currently at hand thus reflect the complexity of the relationship between Ca^{2+} fluxes and bile flow. Indeed, as pointed out by Nathanson (1994), the cellular site at which the increase in cytoplasmic Ca^{2+} occurs may be as important as the extent to which hepatocellular Ca^{2+} is mobilized. Whether a direct correlation between bile flow events and Ca^{2+} oscillatory phenomena (see e.g. Tsunoda, 1993) can be observed with further improvements in resolution, is presently unclear.

The possibility of an optimum state of Ca^{2+} flux for the stimulation of bile flow is further suggested by the observation that the duration of the initial stimulation of bile flow is prolonged by increasing concentrations of Ni^{2+}, but when Ca^{2+} influx is totally inhibited, the flow of bile becomes sub-optimal (Fig.2, Chapter 2). The amount of bile released under conditions of total inhibition of influx is still considerably greater than that observed in the presence of vasopressin alone suggesting that an interaction by the intracellular signals produced in response to these hormones with bile flow mechanisms, in addition to the influx of Ca^{2+}, might be involved in the stimulation of this event. Thus an optimum degree of influx, and possibly Ca^{2+}-cycling rate is suggested for maximum stimulation of bile flow. Taken together, these data indicate that the transient hormone-induced peak in bile flow occurs optimally in conditions where the intracellular Ca^{2+} concentration transiently reaches supra-basal values.

Different thresholds for the stimulation of the transient bile flow events and Ca^{2+} release are indicated by the observation that both the bile flow peak as well as the transient decrease in the bile flow rate subsequent to this peak are observed when 0.05nM vasopressin is infused in the presence of 0.25nM glucagon despite the absence of perfusate Ca^{2+} fluxes with this combination of hormones (Fig.6a, Chapter 3). Since the response is characteristic of that observed at higher concentrations of the hormones, it is possible that some Ca^{2+} is mobilized under these conditions, which, while being able to stimulate the bile flow responses, is not sufficiently large to be detected as efflux in this experimental system. Alternatively, it may reflect an interaction between cyclic AMP and protein kinase C events. In any case, the data point toward the sensitivity of bile flow events to modulation by these hormones, and the sensitisation by glucagon of bile flow induced by vasopressin.

The role of cross-talk in stimulating the flow of bile under these conditions is also evident in the concentration-dependent increase in the peak height in the presence of maximally-stimulating concentration of glucagon. However, it is further evident that in general, any effect of glucagon is to prolong the response to vasopressin, an effect most apparent at low concentrations of the hormones. This enhancement involves both, or either, an increase in the maximum rate of bile flow, as well as the duration of the transient increase. The lack of substantial differences in the bile flow responses in the presence of low concentrations of both hormones indicates that there may be a series of thresholds for these events, with the events described herein falling largely within the same threshold. In fact, the ratio between the concentrations of these hormones appears to be an important determinant of the bile flow rate over the initial few minutes following hormone addition. While the site of action of this effect is not clear, it has been suggested that cyclic AMP may inhibit Ca^{2+}-mediated disassembly of actin filaments (Burgoyne and Cheek, 1987; Hamlin et al., 1990). However, an examination of these responses under conditions of higher resolution may uncover further subtle effects which may point to the mechanisms involved.

It is clear from this work that cross-talk, following the co-administration of glucagon and Ca^{2+} mobilizing agonists, induces a

very different response in bile flow to when they are allowed to act independently (see also e.g., Nathanson et al., 1992a). The extent to which the cross-talk-mediated effects on bile flow result from the increased Ca^{2+} mobilization induced by synergism between the signalling systems, and the extent to which a further synergism between the Ca^{2+} mobilized and the stimulation of components of intracellular machinery are involved in the flow of bile, requires further investigation. Presently, two possibilities stand out as potential sites of interaction of cross-talk between these signalling pathways to stimulate the flow of bile.

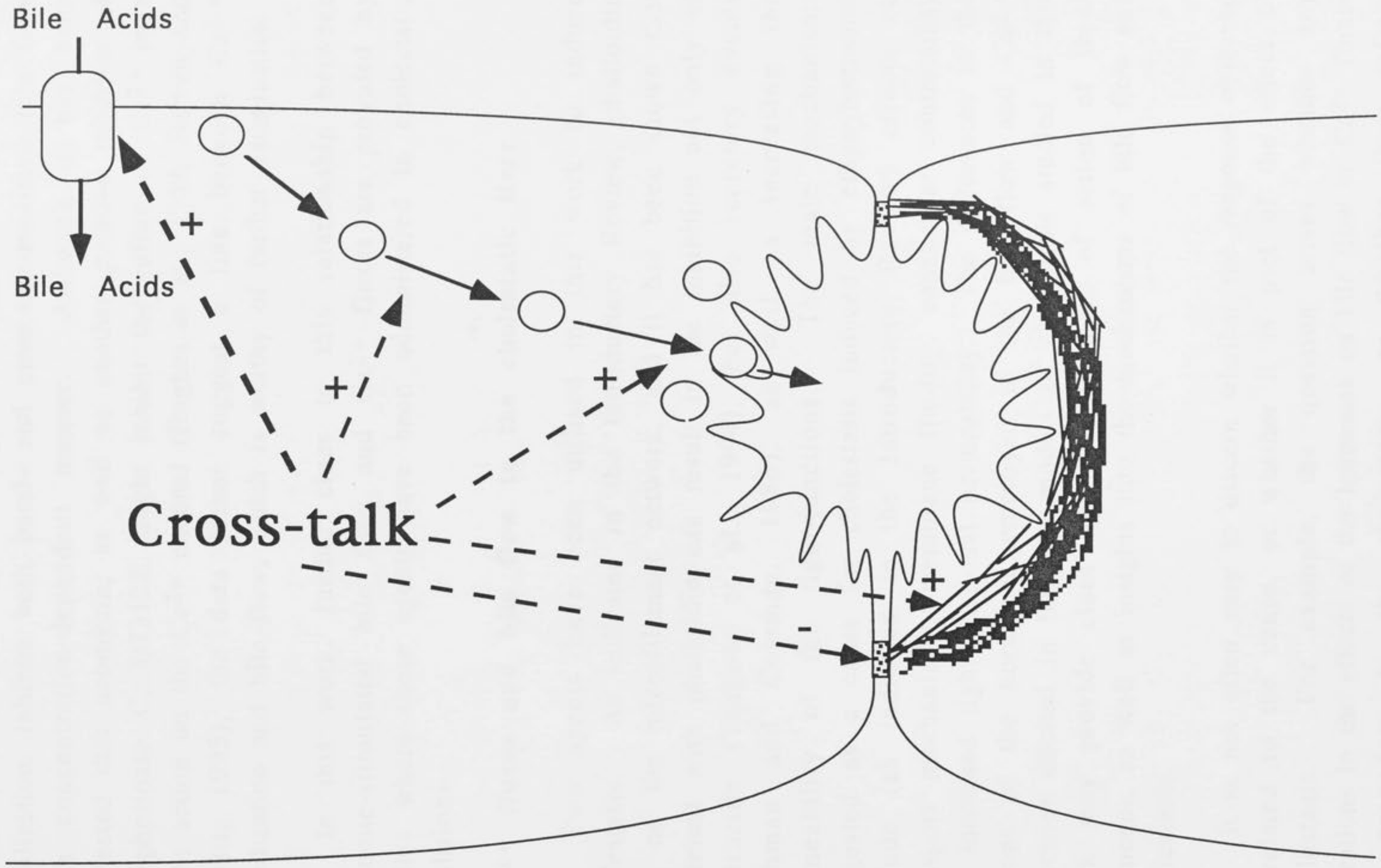
(1) Canalicular contractility induced by increases in cytosolic Ca^{2+} concentration (see e.g. Watanabe et al., 1983, 1988b): The timing of this event correlates with that of the Ca^{2+}-mediated phosphorylation of MLC kinase (Yamaguchi et al., 1991). Therefore the synergistic increase in the peak size could be related to the large increase in intracellular Ca^{2+} concentration in the vicinity of the contractile elements, and the event observed is the result of a sudden contraction of the canaliculus.

(2) Vesicular exocytosis resulting from the increase in intracellular Ca^{2+} concentration: Movement of vesicles from the sinusoidal to the canalicular pole of the hepatocyte are thought to contribute to the flow of bile. This movement of vesicles is stimulated by cyclic AMP (Hayakawa et al., 1990, Hoshino et al., 1993), and this response is further potentiated by Ca^{2+} (Grüne et al., 1993). Furthermore, there is evidence to indicate that the final exocytotic event, whereby the contents of these vesicles are released into the canaliculus, is a Ca^{2+}-mediated event (Burgoyne and Morgan, 1993; Beuers et al., 1993a). Thus there is a stimulation of the movement of the vesicles by cyclic AMP during the initial period of stimulation with glucagon, and as vasopressin is added, the increased cytosolic Ca^{2+} stimulates the transport and expulsion of these vesicles. The events likely to be involved in cross-talk-mediated stimulation of bile flow are summarised in the figure.

The utilization of modulators of components of both the signalling pathways involved, and the putative sites where these act on bile flow processes, should result in further unravelling of the mechanisms of action of these processes, and thereby the

Fig. Points at which cross-talk between the phosphoinositide and cyclic AMP pathways may modulate the flow of bile.

In the presence of cyclic AMP, the uptake of bile acids into the cell is further potentiated by Ca^{2+}. Furthermore, the stimulation of the movements of vesicles by cross-talk between cyclic AMP and Ca^{2+} probably results in an increased release of these into the canaliculus. The enhanced influx of Ca^{2+} under these conditions is probably also reflected in the rate, extent and timing of the contractility of the bile canaliculus. The sites indicated do not exclude the role of individual components of these pathways in these events. Further detail is described in the text.


Bile Acids
Bile Acids
+
+
+
+
Cross-talk

relationships between hormone action. Preliminary data (not shown) indicates that neomycin, a purported inhibitor of phospholipase C (see e.g. Gabev et al., 1989), and thus of Ca^{2+} mobilization, inhibits both basal- and cross-talk-mediated bile flow in a concentration-dependent manner. Although it has been suggested that neomycin, as well as another putative inhibitor of phospholipase C, U73122, might inhibit the influx of Ca^{2+} by a direct action on the Ca^{2+} channel (Hughes et al., 1988; Berven and Barritt, 1995), the data futher suggest a link between Ca^{2+} mobilization and bile flow, which is worthy of further examination.

In this work, further clues to this relationship between hormone-stimulated bile flow and Ca^{2+} fluxes are provided by studies where these agents have been administered in cholestatic conditions.

Ca^{2+} fluxes and bile flow in the cholestatic liver

Two agents have been utilised in this work to induce cholestasis. As indicated in the Introductory Review, phalloidin acts on the microfilament network, and it has been shown that treatment with phalloidin can result in the inhibition not only of contractile (Tsukada et al., 1994), but also secretory events (Rahman and Coleman, 1986), as well as increasing the permeability of the tight-junctions. The main mechanisms suggested as a cause for cholestasis induced by ethynylestradiol include (as indicated in the Introductory Review) effects on transport mechanisms, membrane fluidity, canalicular contractility and increased tight-junctional permeability. The differences in the actions of the hormones employed in the bile flow and Ca^{2+} responses elicited in the two models of cholestasis studied in this work may provide clues to the mechanism of action of these hormones, as well as insights into the mechanisms of bile flow and cholestasis.

It is not often easy to discern whether the response observed is related to the cause, or whether it is part of the effect of cholestasis. For example, the question arises whether any alterations in the effects of the hormones on bile flow or Ca^{2+} fluxes are secondary to the cholestasis, or whether they reflect mechanisms which cause the cholestasis. Under conditions where

phalloidin is used to induce a decrease in the bile flow rate of 50%, no significant effect on the maximum rate of influx, or on the total amount of Ca^{2+} taken up in response to stimulation with glucagon and vasopressin occurs. Thus at least the degree of disruption of the network of microfilaments at the concentration of phalloidin applied, neither a disruption of microfilament-related events (see e.g. Kraus-Friedmann, 1994), nor a decrease in the bile flow rate *per se* are likely to affect Ca^{2+} fluxes induced by cross-talk in hepatocytes. It may be necessary to utilise agents which disrupt microfilaments at higher concentrations than utilised in this study. However, further suggestions that the intracellular location for the interactions may be important (Hajnocky et al., 1994) may arise from work in this thesis which indicates that the cross-talk-mediated Ca^{2+} fluxes are altered in cholestasis.

When cholestasis is induced *in vivo* by ethynylestradiol there is a decrease in the amount of Ca^{2+} taken up in response to cross-talk between glucagon and vasopressin of about 50%. This inhibition of Ca^{2+} influx is significantly greater than the effect on either glucose release or oxygen consumption (see Chapter 4), but similar to the effect on the basal bile flow rate. The locus of this effect is not presently clear, but may indicate that the signalling events are probably not as greatly affected as are the structures involved in the inflow of Ca^{2+} or the flow of bile. Whether membrane proteins which are involved with the events associated with the Ca^{2+} fluxes are affected under these conditions is not clear. Thus the possibility that treatments which relieve the effect on membrane fluidity, such as SAMe (S-Adenosyl-Methionine) might also relieve the effects on hormone-action in cholestatic liver needs to be examined. A decrease of a similar magnitude has been shown on both the flow of bile and Ca^{2+} fluxes in cholestasis induced by T-CDCA (Hamada et al., 1992b; Bygrave et al., 1994). The extent to which impaired intercellular communication might be involved in these events is presently unclear (Fallon et al., 1995).

Another question is whether the decreased Ca^{2+} influx is related to a reduction in the ability to take up the ion, or a decreased clearance from the cytosol. In any case, the severity of the effect of cholestasis on bile flow events appears to be related to the ability to induce Ca^{2+} influx to an extent, and therefore this work lays the foundation for utilizing experimentally-induced

cholestasis as a tool for further elucidating the mechanisms by which Ca^{2+} inflow occurs. Whether changes to the inflow mechanisms have occurred in cholestasis can be examined by utilizing Ni^{2+} and the other inhibitors of Ca^{2+} influx mentioned earlier.

Characteristic of cholestatic liver apparent from this work is the elimination of the initial peak in bile flow in response to vasopressin stimulation (see Chapter 4), as suggested from work with T-CDCA (Hamada et al., 1992b). The effect on the peak induced by vasopressin is considerably less noticeable than the effect on the peak induced by cross-talk (Fig.1, Chapter 4) despite the effect of this agent on basal bile secretion. In fact, no effect of glucagon is observed when infused alone in the presence of phalloidin.

Since the decrease in bile flow rate in response to vasopressin occurs on top of that induced by ethynylestradiol or phalloidin (this work) or T-CDCA (Hamada et al., 1992b), this may indicate that the mechanism by which the decrease in bile flow occurs following vasopressin treatment is different from, or potentiated by, that affected by these cholestatic agents. Furthermore, the fact that there is a greater effect on the increase in bile flow than the vasopressin-mediated decrease in bile flow points to the possibility that even if similar components are involved, the events are mechanistically unrelated. In any case, with ethynylestradiol (this work) or T-CDCA (Hamada et al., 1992b) induced cholestasis, hormone-induced Ca^{2+} fluxes as well as bile flow events are modified.

The decrease induced by glucagon does not occur in any of these models of cholestasis, and therefore indicates that a microfilament-related event may be involved. Furthermore, this observation coupled with the fact that the decrease clearly occurs with vasopressin, indicates that these probably involve different mechanisms. In fact, the possibility is raised that the general decrease in bile flow induced in these models of cholestasis may involve a similar mechanism to that observed with glucagon. This requires further exploration. Since the small Ca^{2+} release normally seen with glucagon does not appear to be affected by the

treatments, the decrease is probably not due to an increase in the intracellular Ca^{2+} concentration.

Another notable change in the effect of glucagon stimulated by ethynylestradiol is that the choleretic activity of the hormone is greatly enhanced (see Fig.1, Chapter 4). Further characterization of this effect is required to enable elucidation of the mechanisms involved. Potentially relevant to this issue is the recent work of Yasumoto et al. (1994) who found Gs expression to be affected by estradiol in MCF-7 cells. They suggested that this might contribute to the increased sensitivity of hormone-stimulated adenyl cyclase activities in this cell line. While to our knowledge similar data with hepatocytes is not available, this point may be relevant to the present findings. If the sensitivity of the hormone-stimulated adenyl cyclase activity is increased by ethynylestradiol treatment, the larger amount of cyclic AMP produced would result in a proportionally greater increase in the bile flow rate. Supporting this are data from present experiments indicating that an increase in bile flow of similar magnitude is produced when a high concentration of dibutyryl cyclic AMP is infused, both in the ethynylestradiol-treated and control liver. The flow of bile increases approximately 2-fold with 500 μM dibutyryl cyclic AMP, as found also by Hamlin et al (1990). Another role for increased G_s expression could be related to its stimulation of endocytosis (Colombo et al., 1994). However, the expression of genes for other proteins which might affect this event is possible through the action of ethynylestradiol, and thus further work is required for their identification.

The effect of bile acids on experimentally-induced cholestasis

- therapeutic potential

Since bile acids modulate both the flow of bile as well as Ca^{2+} fluxes (as discussed in the Introductory Review), studies were undertaken to examine the ability of choleretic agents to restore hormone-induced bile flow in rats in which cholestasis has been induced. Despite studies showing that various bile acids stimulate Ca^{2+} fluxes on their own (see Introductory Review, Section (E) (II)), minimal effects on glucose release, oxygen consumption or

perfusate Ca^{2+} fluxes are observed in the perfused rat liver in response to the bile acids utilised in this work.

The greater amount of Ca^{2+} released into the bile at the stages where bile acids have been added may be due to the bile acids carrying the Ca^{2+} to the bile. Rajagopalan and Lindenbaum (1982) found that the glycine-conjugated bile acid binds calcium more strongly than the corresponding taurine conjugate in either water or buffer, offering a possible explanation for the greater increase in biliary calcium concentration induced by this bile acid (see also Gleeson et al., 1990). The data of Bouscarel et al. (1993) indicate that G-UDCA enters the cell less readily than UDCA or T-UDCA, possibly explaining the lower choleretic potential of this bile acid. Thus part of the biliary calcium changes observed may be due to calcium being transported into the bile by the bile acids. Further insights into this issue should become evident once data is obtained about the relative proportions of 'free' to 'bound' calcium in the bile outflow, especially if measured concomitantly with bile acid levels.

It has been suggested (Beuers et al., 1993b) that Ca^{2+} fluxes in hepatocytes induced by a bile acid like T-UDCA cannot necessarily be regarded as a toxic or cholestatic event. These workers (Beuers et al., 1993b) also observed that T-UDCA had a greater Ca^{2+}-mobilising capacity than unconjugated UDCA in hepatocytes. This would be consistent with our observation in the present study that T-UDCA induced a greater flow of calcium into bile than UDCA. In another study (Beuers et al., 1993a) these workers concluded that the beneficial effect of T-UDCA in cholestasis may in part be related to the Ca^{2+}-dependent stimulation of vesicular exocytosis. Thus the bile acids may be involved in buffering the intracellular Ca^{2+} so that the balance between the stimulatory and inhibitory effects of the ion are appropriately maintained. A direct effect of bile acids on the microfilament network has been shown (Tuchweber et al., 1990). Furthermore, the pericanalicular distribution of F-actin increases in the presence of the cholestatic bile acid taurolithocholate and this effect is partially ameliorated by the co-administration of T-UDCA (Thibault et al., 1993). Recently it has been shown that the stimulation of phosphatidylcholine transfer protein activity by bile acids is involved in the co-ordination of phospholipid secretion into bile (Cohen et al., 1994), and thereby in the regulation of bile

secretion. Furthermore, phosphatidylinositol transfer protein is required for Ca^{2+}-activated secretion (Hay and Martin, 1993), suggesting the possibility that, besides Ca^{2+}, other components of the signalling pathways may also have a role in the control of bile secretion.

The peak height in the presence of the bile acids appears to be increased in proportion to the basal bile flow rate in response to treatment with the hormones. Thus these agents are likely to act by enhancing components of basal bile flow. In order to determine the relative contribution of vesicular and secretory events on the one hand, and contractile events on the other, a first step would involve the utilization of inhibitors of the microtubular system under the conditions described.

An important finding of this work is that while the initial transient cholestatic effect of cyclic AMP is not significantly affected by treatment with the bile acids utilized in this study, the choleretic effect is considerably enhanced both in the cholestatic as well as the control animals. Relevant to this observation is the finding that the Na^{+}-dependent bile acid co-transporter, which is thought to be the most physiologically important bile acid uptake system in hepatocytes (Meier, 1993), is stimulated by cyclic AMP and glucagon (Edmondson et al., 1985; Botham and Suckling, 1986). This effect, which has been attributed to cyclic AMP-mediated hyperpolarization of the hepatocyte membrane (Edmondson et al., 1985), is mediated by protein kinase A and potentiated by Ca^{2+}/ calmodulin-dependent processes (Grüne et al., 1993) but decreased by protein kinase C action (Grüne et al., 1993; Divald et al., 1994).

Thus under the conditions of the study, the bile flow is improved by each of the bile acids utilized, but it needs to be determined whether the lesions which affect the responsiveness to hormones can be ameliorated by bile acid therapy over longer periods of time, and in conjunction with other agents.

Concluding comments and further directions

This work has opened the way to further examination of the mechanisms of Ca^{2+} influx, and their relationship to bile flow in the perfused rat liver.

Immediate future work would involve discriminating between the effects of depletion of intracellular stores from other effects of vasopressin, such as those involving IP_3. In view of the modulatory effect of glucagon in Ca^{2+} mobilization, and the fact that glucagon can mobilize Ca^{2+} under some conditions, the interaction between store depletion and Ca^{2+} mobilization by glucagon alone needs to be examined. In order to localize further the sites of action involved in these events, the role of other components of these signalling pathways downstream from the receptor, for which modulators are available, can be utilised. Of special interest in view of results presented herein are modulators of G-proteins. Concomitant analysis of changes in bile flow induced under these conditions will further advance the knowledge about the relationship between the Ca^{2+} fluxes and bile flow events. Of especial interest would be to examine this effect in cholestatic liver to ascertain the locus of the effect on Ca^{2+} fluxes.

Glucagon has been shown to initiate net influx of Ca^{2+} when the pH of the extracellular medium is increased (Altin and Bygrave, 1987; Bygrave and Benedetti, 1993), and it has been suggested that this feature might be utilized as a tool in studies on the mechanism of Ca^{2+} influx (Bygrave and Benedetti, 1993). Thus utilization of the inhibitors of Ca^{2+} influx mentioned earlier would further enable characterization of the activity of different Ca^{2+}-channel populations. Furthermore, recently evidence has been presented that LU52396 is an inhibitor for Ca^{2+} influx (Clementi et al., 1995) by the capacitative pathway.

Another question for examination is the destination of the Ca^{2+} under these conditions. Where the SERCA inhibitors are utilized, Ca^{2+} should not enter the endoplasmic reticulum, and therefore presumably enter the mitochondria. These organelles appear to have a more important role in hormone-mediated Ca^{2+} fluxes than has been previously thought (see e.g Jouaville et al., 1995), and this question can be examined by utilizing inhibitors of

mitochondrial Ca^{2+} influx, as well as analyses of mitochondrial Ca^{2+} concentration following treatment with the hormones and SERCA inhibitors. The role of the mitochondrial permeability transition pore, which is purported to affect both Ca^{2+} fluxes (Hoek et al., 1995) and bile flow (Roman and Coleman, 1994), can be analysed with appropriate probes to that site.

It has been shown that osmotically-induced changes in hepatocyte volume in the perfused rat liver can result in a transient response in bile flow similar to that observed in this and previous work (see e.g., Bruck et al., 1992; Häussinger and Lang, 1992; Häussinger et al., 1992; Hallbrucker et al., 1992). The extent to which this phenomenon relates to the above observations, however, are unclear at this time. For example, the effects of glucagon (or cyclic AMP) in inducing hepatocyte volume changes were additive with phenylephrine, but synergistic with vasopressin (vom Dahl et al., 1991). In addition to this, phenylephrine alone induced an increase in hepatocyte volume, whereas vasopressin alone induced a decrease. Thus there is scope for further examination of the relationship between cell volume changes, Ca^{2+} fluxes and bile flow. Associated with this is a further examination of the role of the cytoskeleton and other ion movements in cross-talk-stimulated Ca^{2+} fluxes and bile flow.

The extent to which the well known cell heterogeneity in liver lobules contributes to the observations made in this work is difficult to assess at this time. The potential effect of inhibitors of Ca^{2+} fluxes on the propagation of agonist-induced Ca^{2+} waves across the liver lobule (Robb-Gaspers and Thomas, 1994) is unknown. We cannot rule out also for example, the possibility that Ca^{2+} fluxes in cells in a particular location in the liver may be more susceptible to inhibition than in another. This possibility can be minimised by repeating key experiments with retrograde perfusion (see e.g. Jungermann and Katz, 1989). Interactions with other cell types in the liver may also potentially be involved in the regulation of bile flow, in view of reports that prostaglandins are able to modulate bile flow (Beckh et al., 1994), as well as Ca^{2+} fluxes (Altin et al., 1987; Llopis et al., 1993).

The therapeutic potential of bile acids requires examination from the mechanistic aspect. In view of reports that whether the

bile acids is cholestatic or choleretic depends on the concentration at which is infused, it is pertinent to ask whether the transition from choleresis to cholestasis is associated with changes in Ca^{2+} flux characteristics. Furthermore, the finding that bile flow is highly susceptible to modulation by signalling cross-talk, and that this responsiveness is altered in cholestasis provides a sensitive indicator of any aberration in its regulation. Thus, having identified particular markers for cholestasis, agents and conditions which ameliorate the basic lesions present in cholestasis may be sought and identified for therapeutic purposes. Initial steps in these enquiries would involve prophylactic treatment with the bile acids utilised so far, as well as in combination with agents which are known to normalize some of the effects in cholestasis (e.g. SAMe, which improves membrane fluidity).

Indications have been provided in this work that the localization of changes in intracellular Ca^{2+} may be more important than the extent to which these fluxes are stimulated (Nathanson, 1994). This concurs with the suggestion that the frequency of oscillations in intracellular Ca^{2+} concentrations might encode signalling information (Schulman, 1992; Vajanaphanich et al., 1995), and modification of this by cross-talk in secretory cells might encode information which controls the extent of secretion (Vajanaphanich et al., 1995). Thus, as the resolution for the study of these events improves, the relationship between Ca^{2+}-mobilization and bile flow will undoubtedly reveal even further complexity.

Section G

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